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RED PIGMENT PRODUCTION IN APPLES BY MEANS OF ARTIFICIAL LIGHT SOURCES

JOHN M. ARTHUR[1]

INTRODUCTION

This problem was brought to our attention in 1929 by a McIntosh apple grower operating considerable acreage near Kinderhook, New York. The grower pointed out that although a green or red colored McIntosh apple may taste exactly the same there was a difference sometimes of a dollar per bushel in the relative retail market value in favor of the red apples. This study was therefore planned to provide, if possible, a method for developing red pigment in McIntosh apples after the fruit had been picked from the trees in August and September. Red color development in other varieties of apples such as Gravenstein, Northern Spy, and Baldwin was studied in a preliminary way with results very similar to those presented in connection with McIntosh and it is believed that the method may be of quite general application to red pigment production in apples. The characteristic red blush on Elberta peaches was also produced in this way.

On account of the cost of producing color by this method the commercial application is doubtful. It is believed, however, that sufficient progress has been made in developing the present method to warrant publishing the procedure, while application, if any, may be made in the future.

EXPERIMENTAL DEVELOPMENT OF METHOD

In 1929 green McIntosh apples (*Pyrus malus* L.) were placed in each of the spectral glass greenhouses as well as in an ordinary greenhouse and in open sunlight. These houses have been described in detail by Popp (10). The transmission of the glasses used as a roof covering during the present study was:

1. Window glass, transmitting visible region and to wave length 313 mμ in the ultra-violet.

2. Heat-absorbing glass, transmitting visible region and to 313 mμ in the ultra-violet but transmitting only about 10 per cent at wave length 1100 mμ in the infra-red (Fig. 2).

3. Corex A transmitting ultra-violet. Transmits visible region and 80 per cent of the incident radiation at wave length 290 mμ, the solar limit.

[1] The writer hereby gratefully acknowledges his indebtedness to Mr. Paul Judson and Mr. R. G. Hall for furnishing fruit for this study; to Mr. L. C. Porter and other members of the Nela Park Laboratory of the General Electric Company for their assistance in furnishing lamps and energy distribution curves; to Dr. S. H. Eckerson and Mr. W. D. Stewart for their assistance in the microscopical examination of the apple peels; and to Dr. W. J. Youden for his assistance in temperature measurements by means of thermocouples.

4. Blue glass transmitting the visible region except red from wave length 585 mμ to 348 mμ.

5. Amber glass transmitting the visible region from wave length 720 mμ to 472 mμ.

6. Brown glass transmitting the visible region except blue from wave length 720 mμ to 529 mμ. Includes red, orange, yellow, and part of the green.

7. Black heat-transmitting glass with almost no transmission in the visible region but transmitting more than 50 per cent from 1000 mμ on into the longer wave infra-red region[2] (Fig. 2).

After five days under the various filters the apples were observed critically for color. Those held under the third glass with a high ultra-violet transmission were best colored. Apples under glasses 1 and 2 were about equally colored but less in intensity than 3. Blue glass 4 produced faint coloring, while 5 and 6 produced little or no coloring. Apples held under filter 7 transmitting only infra-red developed no color during the 5-day period. The storage under this filter was continued for another three weeks but no trace of color was developed during this period.

From this preliminary experiment it was established that the infra-red of sunlight was of no value in producing the red pigment, ultra-violet was of considerable value and even the extreme ultra-violet of sunlight had considerable effect in producing color in house 3 as compared with house 1 which did not transmit this region. Of the visible spectrum the red-yellow region between wave lengths 472 and 720 mμ was of very little value (house 5) while the blue-violet region limited by wave lengths 348 to 585 mμ had considerable potency in producing red pigment.

Since the ultra-violet region had been found of considerable value in developing pigment, a study was made of the effect of various regions beyond the short wave limit for sunlight using a Cooper Hewitt 220 volt mercury vapor arc lamp in quartz and the same series of filters which was used in a previous study (1). Apples irradiated for 30 minutes with the open arc developed considerable visible injury on the side exposed to the lamp. These apples failed to develop any color when kept in the dark room. When exposed subsequently to sunlight in house 3 under Corex A filter only that part of the apple which did not receive the direct rays from the lamp developed color, as shown in Plate I, A. Apples irradiated for the same time through a filter transmitting one per cent at wave length 250 mμ showed no visible injury but when subsequently exposed to sunlight in house 3, failed to develop the red pigment on the side exposed to the lamp. Development of color is apparently the result of processes going on within the living cells and when these are injured, even though there is no visible sign of injury, failure to develop color is one of the visible results. This will be discussed again in connection with later experiments. The

[2] These filters were supplied by the Corning Glass Works.

experiment also indicates that the extremely short ultra-violet rays beyond the limit for sunlight are not only of no value in producing color but are injurious or lethal to the cells producing the color.

Most of the studies during the autumn of 1930 were made for the purpose of establishing a more nearly ideal source of light for coloring apples.

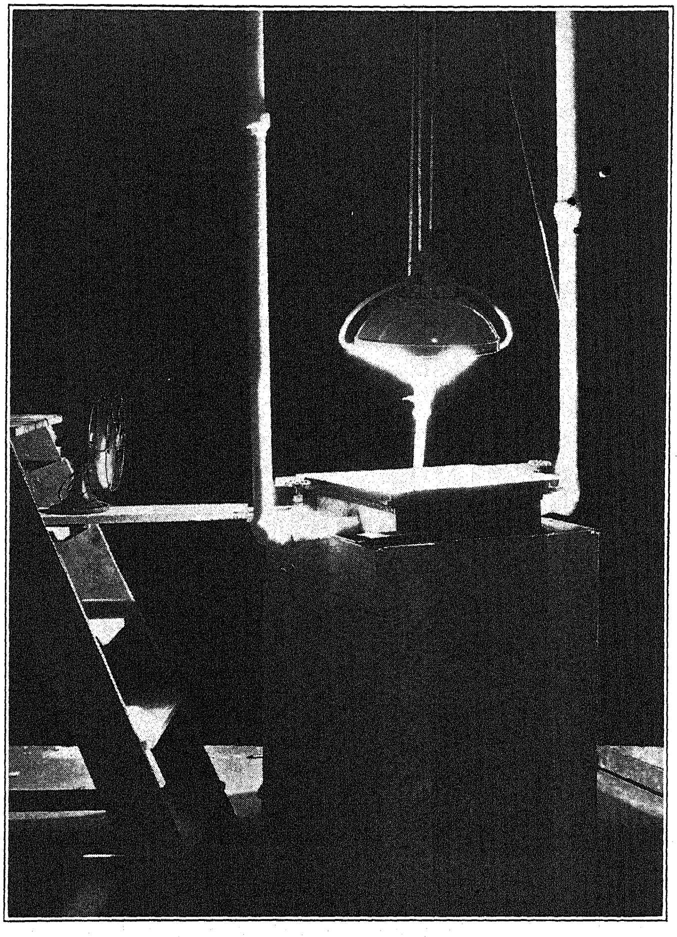

FIGURE 1. Insulated low temperature box covered with black glass filter, showing one of the light sources, the General Electric S-1 lamp, in position.

Sunlight is not dependable and has another disadvantage in that it contains too much infra-red and red and too little ultra-violet to be ideal in quality. A metal box was constructed in which a pipe coil carrying refrigerated brine was installed. The metal box was insulated by placing it inside of a larger wooden box and the space between the walls of metal and

wood was packed with powdered cork. The top was left open. Light filters of various kinds were placed over the open face of the metal box and various light sources were suspended over the filters. A thermostat and an electric heater were placed in the metal box near the tray on which the apples were kept during an experiment. This maintained a given temperature to within a range of 2° C. The box arrangement is shown in Figure 1, with the General Electric type S-1 lamp suspended above the filter.

The following three light sources were used to determine their efficiency in coloring apples, the carbon arc, General Electric type S-1, and the General Electric CX Mazda 500 watt lamp. The carbon arc lamp used was of the twin arc type, 25 ampere and with motor fed carbons. In general, white flame carbons were used so as to approximate sunlight more closely in both quality and intensity of illumination. The General Electric type S-1 lamp combines characteristics of both a filament and tungsten mercury vapor arc lamp sealed into a glass lamp blank very similar to that used in the ordinary Mazda incandescent lamp. The glass used in the construction of this lamp has a high transmission in the short ultra-violet region beyond the limit for sunlight. It has been described in considerable detail by Luckiesh (7) and by Forsythe, Barnes, and Easley (2). The CX Mazda used was an ordinary high-efficiency 500 watt tungsten filament type sealed in a lamp blank having a high ultra-violet transmission. A spectrogram of the emission of this source is shown in Figure 3, number 1.

Apples were colored at about the same rate as in sunlight under each of these light sources. The three types of lamps were chosen originally because of their high output in the ultra-violet region. The intensity of the ultra-violet region reaching the apples was varied by using various filters or by changing the distance of the lamp from the filter.

At about this time a new development appeared in the coloring procedure which was common to all three light sources. As the period of cold storage of the apples after harvest increased it was necessary to expose them for a longer time to produce the same amount of coloring. When first gathered in September they could be well colored by an exposure of three to four days. In October after a period of cold storage it was necessary to expose them to the light source six or seven days. When the fruit was exposed for five days or longer a wrinkled necrotic area developed on the side of each apple exposed to the lamp. This injury was at first attributed incorrectly to the short ultra-violet region, as it had been observed previously that leaves of young tomato plants were injured when exposed to the ultra-violet region immediately beyond the limit for sunlight (1). An experiment was tried with an ordinary 500 watt incandescent lamp placed 30 inches above the apples using a window glass filter over the box. The temperature inside the box was held at 2° C. This arrangement eliminated all of the injurious ultra-violet region but did not prevent injury to the apples.

The typical injury developed again after 5 days' exposure. Since this light source has more than 90 per cent of the total energy output in the infra-red and less than 10 per cent in the visible it was assumed that the injury must be due to the infra-red region.

In order to prove this two experiments were made. The first experiment was to determine the effect of infra-red alone without the visible region. For this purpose the ordinary 500 watt Mazda lamp was used again as a source. This was placed 24 inches above the top of the metal box. A filter was made up of nine pieces of Corning heat-transmitting glass, each six and one-half inches square, set into copper cames so as to form a single composite filter approximately 20 inches square. This was used as a covering for the metal box. The transmission of this glass and curve of output of the lamp are given in Figure 2. The apples were placed about 6 inches below this filter or at a distance of approximately 30 inches from the lamp, and appeared to be in complete darkness. The temperature was maintained at about 2° C. inside of the box. After 5 days' exposure the typical wrinkled, necrotic area developed on the side of the apples exposed to the lamp as shown in Plate I, B. No red color was produced. This apple was exposed with the blossom end toward the lamp. The band of smooth tissue between the two wrinkled areas was produced by pasting a gummed label strip over this area before exposing the apple. The internal temperature of the apple as determined by means of both thermocouples and thermometers was found to be about 20° C. higher than the surrounding air temperature or about 22° C. While this temperature was higher than might be expected it is believed that it was not sufficiently high to have caused the injury on the upper surface of the apple. The injury was probably due to the direct action of infra-red on a tissue which absorbs it freely.

A second experiment was made to determine whether the fruit would be injured by the visible region when the infra-red was absorbed by filters. The same light source was used as in the preceding experiment. A piece of Aklo heat-absorbing glass large enough to cover the metal box was used as a filter. This was set into a wooden frame and made water-tight by means of paint and putty. Water was fed in continuously at one corner of the glass and overflowed at the opposite corner, maintaining a water level over the entire surface of the glass of approximately 1 cm. The transmission of the glass and 1 cm. of water can be seen in Figure 2. The point of maximum output of the lamp is at wave length 950 mμ at which point the water transmits about 80 per cent of the total incident radiation while the glass transmits about 15 per cent. Beyond wave length 1400 mμ the water layer absorbs completely, while from the end of the visible at 700 mμ to 1300 mμ the transmission is limited by the Aklo filter. Apples exposed for five days to an ordinary 500 watt Mazda lamp through this combination of water-glass filter appeared to be completely protected, that is, showed no

EXPLANATION: PLATE I

A. Apple which was exposed on the stem end to a mercury vapor lamp in quartz for 30 minutes, then placed in sunlight under Corex A. It developed color only on the side which had not been exposed to the lethal rays from the lamp.

B. Apple injured by exposure to the infra-red region of an ordinary 500 watt lamp for 5 days. The band of smooth tissue between the two wrinkled areas was produced by pasting a gummed label strip over this area before exposure.

W, P, and C. Apples exposed for 43 hours ending August 31st to mercury vapor arc in Uviol glass with window glass as filter in W and Corex D as filter in C. P was exposed similarly except a mercury arc in Pyrex was used without filter.

F-X through F-15. Apples exposed for 5 days ending September 14th to mercury vapor arc in Uviol using various filters. F-X, cellophane, two layers of which had been soaked in methyl green and one layer in aniline compound. F-9, two layers treated with potassium acid phthalate. F-11, two layers treated with sodium benzoate. F-U, the mercury vapor arc in Uviol without filter. F-3, one layer of methyl violet and one of aniline compound. F-6, one layer of methyl violet. F-15, blue-purple Corex. The transmissions of all of these filters except F-X are shown under the respective numbers in Figure 3. The green crosses were produced by gummed paper strips applied before exposure and show the amount of pigment developed by contrast with this protected area.

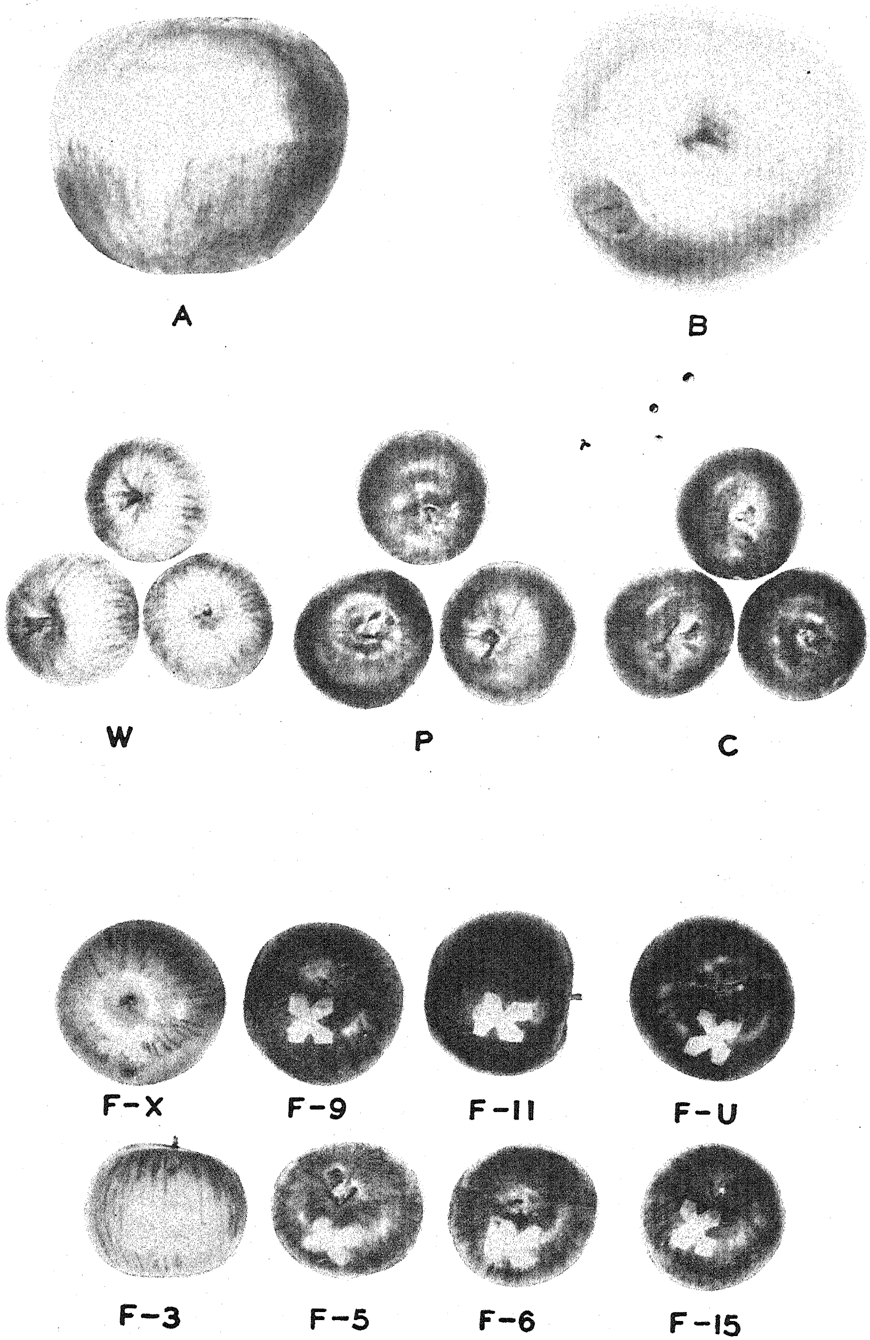

PLATE I—APPLES COLORED BY ARTIFICIAL LIGHT

definite injury or necrotic area on the side exposed to the source. The air temperature inside the box was 2° C. and the internal temperature of the apples 12° C., a rise of 10° C. due to absorption in the visible region and that part of the infra-red which was transmitted by the filter.

From the above studies it was determined that the infra-red region is not only of no value in the coloring of apples but is decidedly injurious. A relatively large fruit such as an apple is well organized for receiving energy in this region but has little or no provision for re-radiating it on account of the low evaporation rate through the peel. This is very different from the

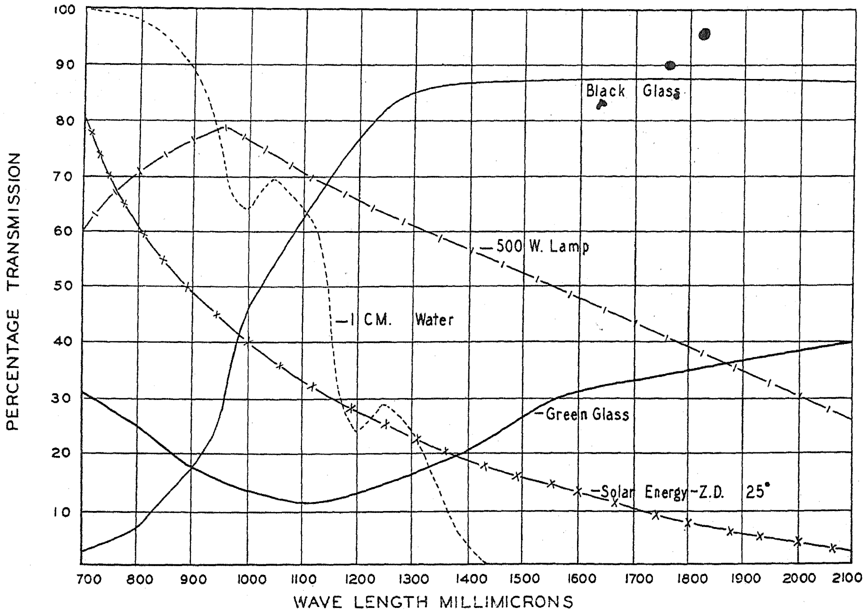

FIGURE 2. Transmission curves of heat-absorbing green glass 2.34 mm. thick and heat-transmitting black glass 3.34 mm. thick. Transmission of one cm. of water and approximate energy distribution curves of a 500 watt lamp and sunlight. The transmission tests of the two glasses were made by the United States Bureau of Standards. The energy curve of the 500 watt lamp was supplied by the General Electric Laboratory, Nela Park, Cleveland, Ohio. The approximate solar energy curve was drawn from Kimball's data (3).

leaf which is admirably adapted to dissipate any such excess energy by the evaporation of water. A tomato plant was placed in the box under the black infra-red transmitting filter and the same 500 watt lamp was used as a source. A thermocouple was inserted in one of the leaves well exposed to the lamp. No perceptible rise in temperature was indicated over that of the surrounding air. As pointed out above, the leaf is an extremely practical receiver and user of energy while the fruit is apparently designed for exposure in sheltered places out of reach of high radiation intensities. The

approximate solar energy curve is also reproduced in Figure 2 from data published by Kimball (4). The amount of infra-red in sunlight is seen to be considerably less than that of the 500 watt lamp due to the higher color temperature of sunlight, yet on account of the high energy value of sunlight there is a similar possibility of injury. Ramsey (11) has noted the injury resulting upon onions exposed to sunlight after harvest and LeClerg (5) has noted an injury on honeydew melons exposed by the killing of leaves by blight. LeClerg attributes the injury to the increased ultra-violet intensity at an altitude of 4000 feet but such injuries may as well be due to the infra-red until definitely shown to be produced by ultra-violet.

From the studies of 1929 and 1930 already described it was apparent that an ideal light source for coloring apples would be one which had little or no infra-red or visible red but which had considerable radiation in the blue-violet end of the spectrum. Such an ideal source should also have a high intensity in the ultra-violet region as far at least as the end of the solar spectrum at wave length 290 mμ or to such a point as would not produce injury to the cells.

The source nearest to this ideal which has been found to date is the mercury vapor arc in either Pyrex or Uviol glass. These tubes were used with good results during the present season of 1931. Since the different lamps vary considerably in the extreme ultra-violet output, they should be placed at a sufficient distance from the apples to produce color at the maximum rate and not injure the tissues. This distance for a lamp of the Pyrex type is approximately 16 inches. For the Uviol tube the distance should be increased or a filter used which transmits little beyond wave length 280 mμ. The first wave length limit in the ultra-violet which will injure a tomato leaf has been determined as being at approximately 285 mμ (1). A similar point in the ultra-violet at which an apple is first injured has not been determined but it is known to be of wave length shorter than 290 mμ. During the 1931 study a Cooper Hewitt Uviol tube of the industrial type, 50 inches in length, was used. The tube was fitted with a mat surface aluminum reflector which had a high reflection in the ultra-violet region. This was operated in a cold room held at an air temperature of 15° C. At a distance of 16 inches from this tube the internal temperature of the apple was observed to be 21° C., a rise of 6° C. due to absorption in the visible and infra-red region. This temperature seems to be the best for coloring apples and at the same time preventing undue softening and ripening of tissues. McIntosh apples can be well colored in a 48-hour exposure under these conditions if picked early in the season and exposed during the first week of storage. They do not ripen appreciably during the coloring process and are as green to the taste after color has developed as when first exposed.

The rate of coloring and also the final amount of pigment produced decreases with increased time of storage after picking. It is difficult to de-

velop any considerable amount of color on stored apples by exposure to the arc after November 15th. By January 1st no color could be produced on apples taken from a commercial storage plant. It is important to harvest apples as early in the season as possible in order to get rapid color development. Apples picked on August 25th colored very well in 40 hours when exposed to the mercury vapor lamp in Uviol using Corex D as a filter at an air temperature of 15° C. Apples picked on September 19th were colored to the same degree only after 96 hours' exposure under similar conditions. The apples in the first case were too green to be palatable. In the second case they were in the normal condition for commercial harvesting, palatable, but still much more acid than the usual ripe McIntosh apple, and very green in color. It is apparent, therefore, that the time for producing the same intensity of color had increased from 40 hours to 96 hours during the period August 25th to September 19th, with no visible loss in chlorophyll pigments. The production of pigment, therefore, does not seem to be related to chlorophyll decomposition.

Since the pigment is developed in the cell sap of the cells in the peel of the McIntosh apple, experiments were made to determine whether the peel alone was sufficient to develop color. Thin pieces of peel about one-half inch in diameter were removed from green colored apples. These pieces were floated upon water in small beakers and exposed to the lamp. The pigment developed at the same rate and to the same intensity as if it were still attached to the apple. If the peel was ground with sand in a mortar previous to exposure or if it was heated or treated with 95 per cent alcohol so as to injure or kill the cells, no pigment developed. It was pointed out above that ultra-violet of very short wave length injured or killed the cells so that no pigment developed. Production of the pigment is a function of the living cells; dead cells have no power to form pigment. This does not, however, preclude the possibility that dying cells may produce pigment while they are slowly dying; it indicates only that the cells must be living to start with.

The possibility that the pigment may be produced from colorless compounds previously existing in the cells was also considered. The red pigment can be easily extracted from the cells by means of hot water rendered slightly acid. When this is evaporated to dryness the pigment can be recovered comparatively free from contaminating material by means of absolute alcohol. This extraction was tried with green apple tissue and the extract was exposed to the lamp. No color developed. Pressed and filtered juice from green apple peel similarly exposed developed no color. So far it has been impossible to show that green cells contain any pre-existent compound which will form the pigment when exposed similarly to the living green peel.

On November 8, 1931, McIntosh apples were removed from storage at

5° to 8° C. and were examined microscopically. These apples had been in storage since September 15th. The peels were much easier to separate by means of scalpels from the underlying tissue than when they were first put into storage. The cell contents of the inner layers of the peel were collected more or less into large aggregates and most of them could not be plasmolized with salts. They gave every indication of being, in the main, dead and disorganized tissue. Some apples of the Northern Spy variety were obtained on November 15th from a local commercial cold storage plant. These apples were selected from several cases as being the finest in size and greenest in color. They were also as free as possible from bruises. The flesh was very firm and still had the highly acid taste of a half-ripe apple. The cells in the peel of these apples appeared very similar to those of the McIntosh variety. A few cells, especially around the blossom end, appeared normal but the great majority appeared to be dead and disorganized. The fact that the cells of the peel are dead, it is believed, accounts for the inability of green apples to produce color when exposed to the lamp as it has already been shown that only the living peel has the power to produce the red pigment.

EFFECT OF TEMPERATURE ON COLORING

As pointed out above, the best room temperature for coloring is approximately 15° C. This results in an internal temperature of the apple of 21° C. when placed at a distance of 16 inches from the tube. Color was developed at slightly less than half of this rate when the air temperature was held at 5° C. That is, it was necessary to expose apples more than twice as long at this temperature to get the same amount of coloring as at a 15° C. room temperature. Apples will color apparently no more rapidly when held at a temperature higher than 15° C. and since they ripen and soften much more rapidly at a higher temperature this seems to be best for practical color development.

COLOR PRODUCTION UNDER VARIOUS FILTERS

Various kinds of filters were used with the mercury vapor lamp in Uviol glass as a source in order to determine the region most effective in producing color on apples. The filters were mainly of two types, glass having very sharp transmission limits or cellophane soaked in different dyes or other chemical compounds as described by Withrow (13). The glass filters have the distinct advantage in that they are more permanent and in general have much sharper limits of transmission than cellophane. The cellophane filters are useful in obtaining a high ultra-violet transmission with abrupt absorption bands at various regions in the visible or ultra-violet depending upon the dye or chemical used to impregnate the cellophane. The filters were chosen with two ideas in mind; first, to determine the ultra-violet

wave length limits at which pigment could be produced without injury to the apple tissue, and second, to determine the wave lengths most effective in producing pigment whether in the ultra-violet or visible regions. The transmission spectra of the filters used are shown in Figure 3. Spectrograms

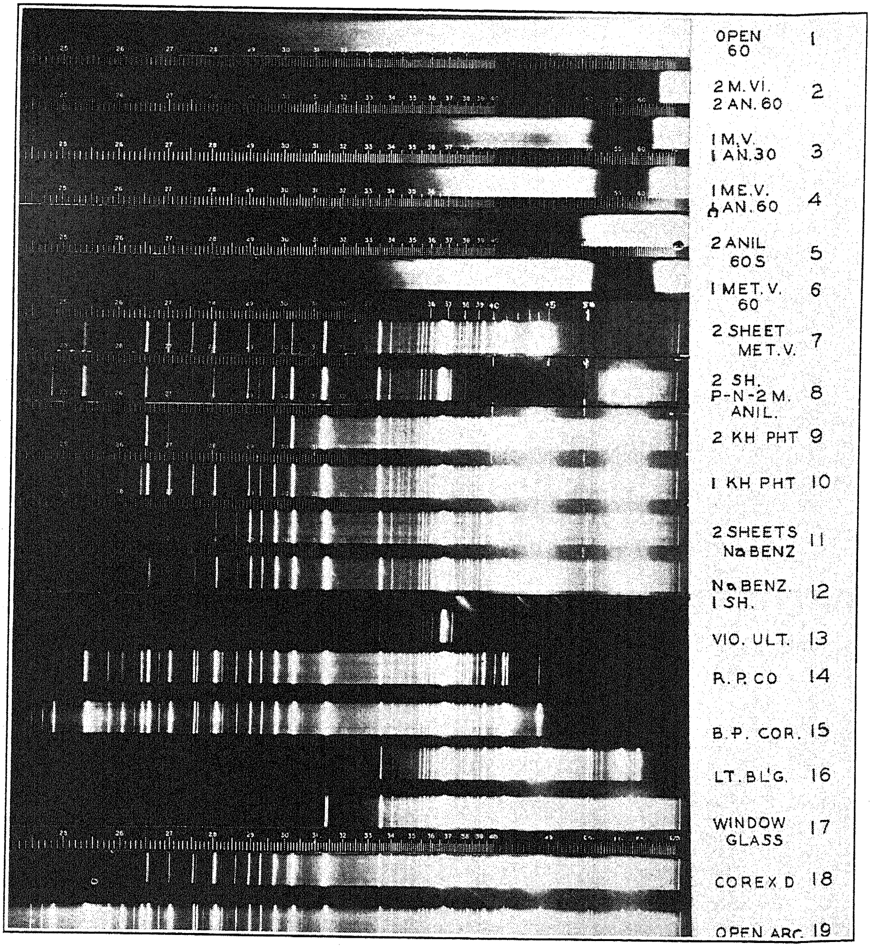

FIGURE 3. Transmission of filters used in the study. 1. CX Mazda lamp without filter, 60 seconds' exposure. 2. Two layers of cellophane soaked in p-nitrosodimethylaniline and two layers soaked in methyl violet, exposure same. 3. One layer of each dye, 30 seconds' exposure. 4. Same as 3, except 60 seconds' exposure. 5. Two layers of p-nitrosodimethylaniline, 60 seconds' exposure. 6. One of methyl violet, 60 seconds' exposure. 7. Two of methyl violet. 8. Same as 5, except mercury vapor lamp as source. 9. Two layers of potassium acid phthalate. 10. Same as 9, except one layer. 11. Two layers of sodium benzoate. 12. Same as 11, except one layer. 13. Violet-ultra, Corning. 14. Red-purple, Corex. 15. Blue-purple Corex. 16 Light blue-green, Corning. 17. Ordinary window glass. 18. Corex D. 19. Mercury arc in quartz.

1 through 6 were taken with a CX Mazda lamp as a light source and numbers 7 through 19 were taken with a mercury vapor arc in quartz as a light source. The first 12 spectrograms show the transmission of cellophane soaked in various organic solutions while the last 7 are taken through various glass filters used in the study. The cellophane filters were made up by first dipping cellophane in the organic material, allowing it to dry, and then stretching one or more layers over windows cut out of cardboard box lids. The box lids were then inverted over apples and placed under the lamp. The glass filters were made similarly by mounting pieces of the various glasses about six and one-half inches square in cardboard box lids. Filters 17 and 18 were not mounted in this way but were placed directly over the fruit. These filters were pieces of window glass and Corex D about twelve inches square. Filters 3, 4, and 6 contained one layer of cellophane soaked in methyl violet. Filters 2 and 7 contained two layers of this material. These filters have a strong absorption band from wave lengths 500 to 615 mμ and a weak general absorption of energy from wave length 350 mμ toward the shorter wave lengths, although the strong ultra-violet lines are transmitted as shown in 7. Filters 2, 5, and 8 each contained two layers of cellophane soaked in *p*-nitrosodimethylaniline and numbers 3 and 4 contained one layer. This dye has a weak absorption band between wave lengths 410 and 460 mμ. Uhler and Wood (12) using the dye alone observed that all lines between wave lengths 324 and 363 mμ are transmitted with almost no decrease in intensity. When four layers of cellophane are used, two of which have been soaked in methyl violet and two in *p*-nitrosodimethylaniline, all of the spectrum is absorbed up to wave length 640 mμ as shown in the spectrogram of filter 2. Filter 6 consisting of one layer of methyl violet slowed down the rate of pigment production. Only a weak striping of red was produced after 5 days' exposure to the lamp. Filter 5, containing two layers of the aniline compound, protected to about the same extent, while a single layer of each dye protected almost completely; only a faint striping was produced. Two layers of each dye (filter 2) protected completely. Another cellophane filter, the transmission of which is not shown in the spectrograms, was made up of two layers soaked in methyl green and one soaked in the aniline, was used. This also protected almost completely. The green dye absorbs in the red, violet and ultra-violet regions. This was called filter X. The amount of pigment formed under the various filters is illustrated in Plate I, F-X, F-9, F-11, F-U, F-3, F-5, F-6, and F-15. The apples are numbered with the corresponding filter numbers used in the spectrograms (Fig. 3). F-U is the control under the open mercury arc in Uviol (Fig. 4, No. 2). The green crosses were made by pasting paper crosses on the green apples before exposing them to the lamp and show the amount of pigment developed during the exposure by contrast with the protected area. In Figure 3, spectrograms 9

and 10 represent filters having two and one layers respectively of cellophane impregnated with potassium acid phthalate. Filters 11 and 12 are similar except that two and one layers of sodium benzoate were used. Filter 9 was made up according to the method of Withrow (13) and is the same as filter number 2 used by him. Filter 11 is the same as the number 1 filter used by Withrow who published the transmission curves of these two filters (13). He found that the phthalate filter had a very sharp ultra-violet limit of wave length longer than 290 mμ. It is believed that this sharply defined limit is due to an absorption band which appears between wave lengths 295 and 275 mμ, and that in reality this filter transmits more energy at wave length 265 mμ than the sodium benzoate filter used by Withrow. This is apparent by a comparison of spectrograms 9 and 11. Photographs of apples colored by exposure through these two filters are shown in Plate I, numbers F-9 and F11. The sodium benzoate filter gave a slightly better color to the apple. Since both filters have apparently an equally high transmission throughout the visible region this is no doubt due to the higher transmission between 290 and 295 mμ. Apples colored by exposure through Corex D (filter 18) and window glass (filter 17) show this same effect; that is, much more pigment is produced under the glass having the higher transmission near wave length 290 mμ in the region near the limit for sunlight. These apples, after an exposure of 43 hours ending August 31st, are shown in Plate I, W and C, while P shows the amount of color produced under a Pyrex tube in the same time. Considerable protection against pigmentation is afforded by window glass when apples color rapidly as shown in Plate I, W. Corex D was found to be an ideal filter to use with the mercury arc in Uviol glass. These apples produced maximum color as shown in Plate I, C. Spectrograms of this lamp are shown in Figure 4, number 2, and number 1 in the same figure was made by using the lamp as a source and Corex D as a filter. This arrangement gives considerable energy as far as 290 mμ and yet does not transmit sufficient energy beyond this point to produce an injury on the cells of the apple. The mercury vapor arc in Pyrex also produced good pigmentation as shown in Plate I, P but not quite equal in intensity to the arc in Uviol with Corex D filter. All of the colored glass filters shown in spectrograms 13 through 16 gave better protection against pigmentation than clear Corex D (Fig. 3, No. 18). Energy transmitted through Corex A blue-purple (number 15) produced the greatest amount of color, with light blue-green next (number 16) and red-purple Corex A (number 14) the third best. A photograph of an apple exposed under Corex A blue-purple (number 15) is shown in Plate I, F-15. Using Corning Violet-ultra (number 13) only a faint striping was produced. Exposures through all of these filters were made in a five-day period ending September 19th on fruit picked September 9th. From these results it is believed that the visible spectrum from

wave lengths 600 to 400 mμ and the ultra-violet region from wave length 400 to at least 290 mμ is effective in producing pigment. Of these regions the short ultra-violet near the limit for sunlight which is not transmitted by ordinary window glass is especially effective.

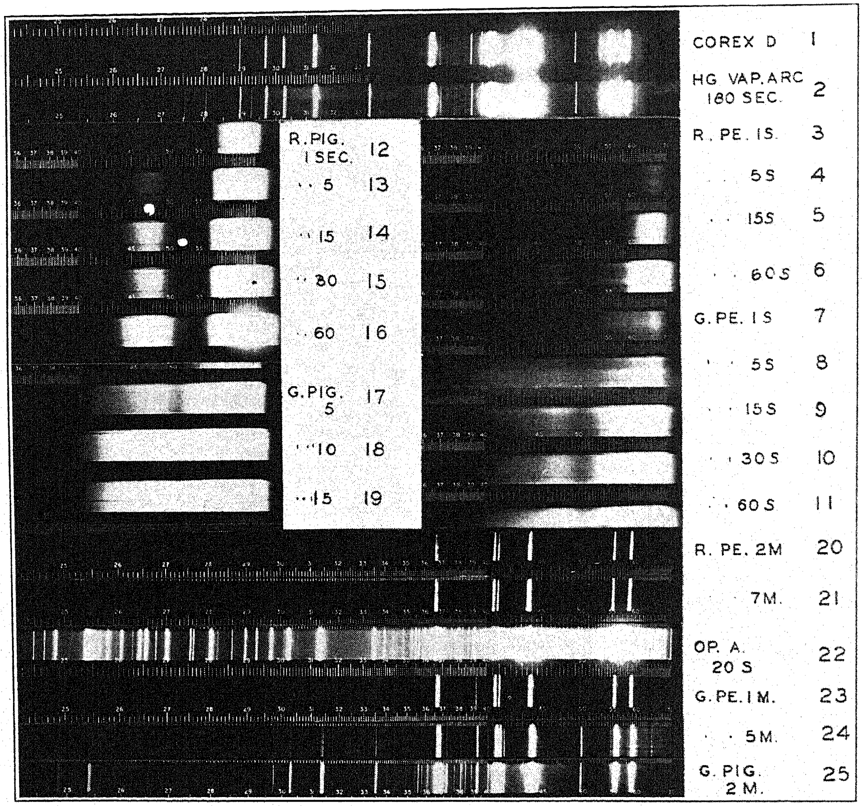

FIGURE 4. Transmission spectra of green and red apple peel and extracted pigments 1. Mercury vapor arc in Uviol as source with Corex D filter. 2. Same as 1, except without filter. 3 to 6. Red peel 1, 5, 15, and 60 seconds' exposure. Source CX Mazda. 7 to 11. Green peel, 1, 5, 15, 30, and 60 seconds' exposure on the same plate as 3 to 6. Source CX Mazda. 12 to 16. Extracted red pigment 1, 5, 15, 30, and 60 seconds' exposure. Liquid layer thickness 1.2 cm. Source CX Mazda. 17 to 19. Extract of green peel 5, 10, and 15 seconds' exposure. Source CX Mazda. 20 to 21. Red peel 2 and 7 minutes' exposure. Source mercury vapor arc in quartz. 22. Mercury vapor arc in quartz without filter, 20 seconds' exposure. 23 to 24. Green peel 1 and 5 minutes' exposure taken on same plate as 20, 21, and 22 and comparable as to intensity of lines. 25. Extract of green peel 2 minutes' exposure.

TRANSMISSION OF GREEN AND RED APPLE PEEL

Only those wave lengths which are absorbed by the peel can be effective in producing color. A study was therefore made by means of a quartz spec-

trograph of the transmission of both the green and red peel and the extracted pigment. For this study very thin sections of the peel about 2 cm. in diameter were mounted between two clear quartz plates. A sheet of tinfoil with a hole in the center about 1 cm. in diameter was pasted over one of the plates. The hole in the foil was centered on the section of peel and the plates were mounted in front of the spectrograph so that only the light coming through the hole illuminated that part of the peel directly in front of the slit. Extracts of red and green peels were made as already described by extracting with boiling water, filtering, evaporating to dryness and dissolving out the pigments with absolute alcohol. The extracts for the transmission tests were placed in a small quartz cell with parallel walls. The thickness of the liquid layer was 1.2 cm. The spectrograms are shown in Figure 4. Numbers 3 to 11 were taken on the same plate and show the relative transmission in the visible region of the red peel, in numbers 3 to 6, and the green peel in numbers 7 to 11. Spectrograms 12 to 16 show the transmission of the extracted red pigment in the visible region and numbers 17 to 19 show the transmission of the extracted green pigment. Numbers 20 and 21 show the ultra-violet limits of transmission of the red peel and numbers 23 and 24 show similarly the limits of the green peel. Number 22 is the mercury vapor arc in quartz. Number 25 shows the ultra-violet transmission of the diluted green pigment. Numbers 20 to 24 were taken on the same plate and are therefore comparable as to intensity of various lines. The exposure time in minutes (M) or seconds (S) is indicated on each spectrogram. A comparison of numbers 3 to 6 with numbers 7 to 11 shows that the red peel is much more opaque to all wave lengths than the green. Five seconds' exposure (number 4) through the red peel is not equal in intensity to one second (number 7) through the green peel in the red region where both are most transparent. Similarly a comparison of the two lines near wave lengths 492 and 334 mμ in numbers 21 and 23 shows that the red peel exposed for 7 minutes transmits less than the green, number 23, exposed for one minute. The transmission of the green peel is, therefore, more than seven times that of the red in both of these regions. The red peel has a strong absorption band in the green-yellow region between 490 and 560 mμ (number 6) and it absorbs to a great extent the whole ultra-violet and visible regions up to wave length 600 mμ. The absorption band between wave lengths 490 and 560 mμ is in the region which is characteristic of some anthocyanin pigments. Robinson and his coworkers (3) have given the range of absorption of both natural and synthetic chrysanthemin chloride and find this to be between wave lengths 480 and 570 mμ. The red peel has a limit of transmission in the ultra-violet at wave length 297 mμ. The last line appearing on the print in number 21 is at 302 mμ. The original plate was exposed for 7 minutes. The green peel transmits as far as wave length 253 mμ although the last line appearing on

the print in number 24 is at 297 mμ. This was a 5-minute exposure. The green peel has no absorption bands in the visible region. The apparent band in numbers 8 to 11 and again in the extracted green pigment (number 17) centering at wave length 500 mμ is due to the lack of sensitivity of the Cramer plate in this region. This has already been pointed out by Luckiesh (6, p. 16). The green pigment has a weak absorption band centering around wave length 280 mμ in the extreme ultra-violet (number 25) but the green peel did not show this band definitely (number 24). The transmission tests were made mainly during November after a period of storage. It is possible that the transmission has changed slightly from the original condition at picking time.

DISCUSSION OF RESULTS

Magness (8) found that Jonathan apples were colored by exposures of one hour each day to "dilute ultra-violet light." Greater exposures injured the fruit. He observed that fruit did not color as rapidly when exposed to sunlight through window glass as when exposed directly. Fruit picked October 5th in Washington started to develop color after 2 or 3 days' exposure to sunlight while if it was stored for two weeks it required a full week of exposure to sunlight before any color developed. These observations agree with the results reported herewith. It is important to expose the apple early in the season for best color development. The last week in August in this region seemed best. The reason for this is believed to be due to the rapid death rate of the cells of the peel shortly after picking or as the apple matures and dead or injured cells were shown to produce no pigment. Magness also found that apples stored at 30° F. for two weeks produced more pigment than those stored at 45° to 50° F. Evidently lower temperatures prolong the life of the cells.

More recently Pearce and Streeter (9) have studied the development of color in apples under various filters using mainly sunlight as a source. They also exposed apples twice daily for nine days to a mercury vapor lamp (Alpine sun lamp) at a distance of 30 inches. They found that the apples were severely injured and that contrary to Magness' observations the ultra-violet did not produce color. When sunlight was used they found by means of various filters that the region between wave lengths 360 and 450 mμ was effective in producing color with an optimum at 410 mμ. They conclude that Pyrex and window glass will not interfere with color formation because both transmit this region completely. The reason for their failure to get color development in the region of sunlight which is not transmitted by window glass was, no doubt, due to the low intensity of this region in sunlight. Since autumn sunlight has little or no energy in the region from wave lengths 312 to 290 mμ no difference would be apparent when a glass filter transmitting to only 312 mμ was imposed. Their failure

to get good color development under the Alpine lamp was, no doubt, due to their admitting the short wave lethal region beyond wave length 290 mμ. The injury of this region as well as that of the infra-red have both been discussed in the present paper and the importance of removing both of these regions for practical color development can not be over-emphasized. As already pointed out the mercury vapor arc in Uviol is the best light source studied for producing color as it has little infra-red and a high output in the violet and ultra-violet while the lethal region beyond 290 mμ can be easily removed by means of Corex D or other filters. It has the additional advantage that its operation in a cold storage room adds very little heat to the general air temperature of the room, and in order to prevent softening of the fruit it is important to preserve a low storage temperature.

SUMMARY

1. Preliminary trials with colored glass filters and sunlight as a source established the fact that ultra-violet, violet, and blue regions of sunlight were most valuable in producing color on apples after they had been picked.

2. Work done with artificial light sources such as the carbon arc, CX Mazda, and S-1 lamps showed that these sources were all effective in coloring apples but all produced severe injuries on apples due mainly to excessive energy in the infra-red region. Using a black glass which transmitted no light but only infra-red, apples were burned by a 500 watt lamp at a distance of 30 inches and at an air temperature of 2° C. in an exposure period of 5 days. The internal temperature of the apple was found to be about 22° C.

3. The best light source for coloring apples was found to be the 50-inch mercury vapor arc in Uviol glass, placed at a distance of about 16 inches from the fruit and used in conjunction with a Corex D filter. The best air temperature was found to be 15° C.

4. The rate of pigment production when exposed as above was greatest on fruit picked green on August 25th. This fruit was well colored after 40 hours' exposure. The rate of color production fell off from August 25th in fruit either picked or left upon the tree.

5. The green peel when removed from apples and floated upon water exposed to the lamp colored at the same rate as when left intact. When the peel was heated or placed in alcohol or otherwise treated so as to kill or injure the cells it would not color. Production of color was found to be a function of the living cells.

6. The reason that fruit does not produce color after a period of storage is believed to be due to the death of the cells in the peel. Most of the epidermal cells were found to be dead on apples stored until November 8th.

7. Using various filters and the mercury lamp as a source the region

producing the pigment most effectively was found to be the short ultra-violet from wave lengths 312 to at least 290 mμ and the visible region from wave length 600 mμ to the beginning of the ultra-violet.

8. The transmission of the green and red peel and extracted green and red pigments was determined and is discussed herewith.

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GERMINATION OF BAYBERRY SEEDS

LELA V. BARTON

INTRODUCTION

Treatment at various low temperatures for different lengths of time has been found effective for overcoming dormancy in the seeds of a number of different plants. Many rosaceous forms give good germination after such treatment (Crocker 3, 4, 5; Crocker and Barton 6; Davis and Rose 7; Flemion 8; and Harrington and Hite 9).

Seeds of many coniferous trees also react favorably to low temperature stratification (Barton 1, 2; Pack 10) although the degree of dormancy in different species varies greatly.

Rose (11) describes low temperature treatment as a means for after-ripening seeds of *Tilia* and *Sambucus*.

Seeds of the bayberry (*Myrica carolinensis* Mill.) germinate very poorly or not at all if they are planted in a flat and kept at a greenhouse temperature of about 70° F. Experiments were conducted to determine whether low temperature treatment previous to planting would overcome this dormancy and whether the presence or the absence of the waxy outside coating had any effect on germination or the retention of vitality in dry storage.

METHOD

Seeds were collected from the Institute grounds November 5, 1928. Each seed is enclosed in a fruit which is covered with a layer of wax. For the tests reported here some of the seeds were left with the wax intact. These are referred to as "waxy" seeds. The wax was removed from other seeds by using a Hobart machine equipped with a wire stirrer. These are referred to as "cleaned" seeds. Five hundred each of waxy and cleaned seeds were planted in flats immediately and put in the greenhouse and in open, mulched, and board-covered cold frames. At the same time 100 seeds each were put in moist granulated peat moss in electrically-controlled ovens at constant temperatures of 15°, 20°, 25°, 30°, and 35° C. and at daily alternating temperatures of 10° to 30° C. and 20° to 30° C., and 500 seeds each were put in peat moss at constant temperatures of 1°, 5°, and 10° C. From the latter stratification samples of 200 seeds each were planted in the greeenhouse after two and three months at the low temperatures.

Another lot of seeds was collected from the same plants in September 1930. These seeds were used to make tests of the extent and rate of after-ripening at 1°, 5°, and 10° C. when the seeds were fresh and also after dry storage in the laboratory for 1, 2, 3, 4, 5, 6, 7, 9, and 10 months. Here again part of the seeds were cleaned and the others were left with the wax intact.

A mixture of one-third soil, one-third sand, and one-third peat moss was used for all of the flat plantings.

RESULTS

The results of the tests with seeds of the 1928 crop are shown in Tables I and II. From Table I it will be seen that fall planting is effective in producing a good stand of seedlings (up to 70 per cent) from waxy seeds in the spring, especially if the board-covered cold frame is used. The open frame was subject to all the temperature fluctuations of this locality. The board-covered frame was more protected but still allowed rather wide variations of temperature while the temperature under the mulch remained above freezing. The mulched condition proved less effective than the board-covered in spite of the fact that the temperature there was nearest 5° C., which was found by stratification tests in constant temperature ovens (Table II) to be equal if not actually superior to 1° C. for after-ripening. The explanation for this apparent discrepancy is to be found in the fact that germination begins at 5° C. after two months. This means then that in the cold frame the young seedlings probably appeared under the mulch some time in February and were killed before the mulch was removed. Germination starts later at lower temperatures and hence the seedlings under the board cover did not appear too early. There was poor seedling production in the open frame.

TABLE I

GERMINATION OF FRESH SEEDS OF MYRICA CAROLINENSIS PLANTED IN FLATS NOVEMBER 9, 1928; 500 SEEDS PER SAMPLE

Temperature		Outside of seed	Germination percentages in June 1929
Greenhouse 68° to 70° F.*		Waxy	1.0
		Cleaned	1.2
Cold frame	Open	Waxy	23.5
		Cleaned	19.2
	Mulched	Waxy	35.4
		Cleaned	35.6
	Board-covered	Waxy	70.0
		Cleaned	66.4

* During winter months.

On the other hand plantings made in a greenhouse in November gave only 1 per cent seedling production by June of the next year (Table I). There was no appreciable difference in the germination of waxy and cleaned seeds.

From Table II, it will be seen that either 1° or 5° C. for a period of three months was effective for after-ripening waxy or cleaned seeds; samples from these conditions gave seedling productions of 81 to 88 per cent when planted in greenhouse flats. However, after-ripening proceeded more rapidly at 5° C. especially for waxy seeds since two months at that temperature resulted in 69 per cent germination from greenhouse plantings while two months at 1° C. gave only 37 per cent germination.

TABLE II
GERMINATION OF FRESH SEEDS OF MYRICA CAROLINENSIS (1928 CROP) IN THE GREENHOUSE AFTER STRATIFICATION FOR 2 OR 3 MONTHS AT 1°, 5°, AND 10°C.; 100 SEEDS PER SAMPLE

Stratification temperature	Outside of seed	Germination percentages after stratification for	
		2 months	3 months
1° C.	Waxy	37	81
	Cleaned	65	83
5° C.	Waxy	69	83
	Cleaned	75	88
10° C.	Waxy	30	48
	Cleaned	37	60

No seedlings were produced at constant temperatures of 20°, 25°, 30°, and 35° C. or at daily alternating temperatures of 10° to 30° C. or 20° to 30° C., in spite of the fact that this experiment was allowed to run for six months. A few seedlings were produced at 15° C. in five months (waxy seeds produced 2 per cent, cleaned seeds 29 per cent).

More detailed tests of the extent and rate of after-ripening at 1°, 5°, and 10° C. were begun in September 1930 with seeds collected from the same source. Repetition of the tests at intervals of one or two months up to ten months furnished material for observation of the effects of dry storage and the removal of the wax on germination and vitality. The results of these tests are shown in Table III. All of the germination percentages reported are from sample plantings made in the greenhouse after intervals of one, two, three, or four months at 1°, 5°, or 10° C. Seedlings began to appear 5 to 15 days after planting in the greenhouse and germination was complete in 15 to 25 days. The duration of each test was 30 days.

It will be seen that fresh seeds both cleaned and waxy germinated best (85 and 78 per cent) after three months at 5° C. The cleaned seeds, however, gave good germination after two months at 1° or 5° C. and after three or four months at 1° C. In many cases after three months' stratifica-

TABLE III

GERMINATION PERCENTAGES OF WAXY AND CLEANED SEEDS OF MYRICA CAROLINENSIS IN THE GREENHOUSE AFTER STRATIFICATION FOR 1, 2, 3, OR 4 MONTHS IN ACID PEAT AT 1°, 5°, OR 10° C.; 200 SEEDS PER SAMPLE

Months of dry storage	Outside of seed	Stratification temperature, and time in months											
		1° C.				5° C.				10° C.			
		1	2	3	4	1	2	3	4	1	2	3	4
Fresh	Waxy	0	7	56	71	0	39	78	77	0	0	11	13
	Cleaned	1	75	76	77	0	84	85	—	0	20	54	—
1	Waxy	—	12	71	79	—	64	84	79	—	1	6	39
	Cleaned	—	28	61	75	—	63	92	55	—	4	36	—
2	Waxy	2	28	71	86	9	80	84	—	2	1	48	—
	Cleaned	3	28	59	67	28	69	78	—	2	10	61	—
3	Waxy	0	15	82	83	3	48	84	81	1	6	40	71
	Cleaned	0	19	68	48	0	63	76	—	1	22	77	—
4	Waxy	0	56	72	—	0	41	79	—	0	6	63	—
	Cleaned	0	59	72	—	1	61	77	—	0	42	80	—
5	Waxy	0	31	80*	—	2	44	87	—	1	4	54	—
	Cleaned	0	26	61*	—	1	72	72	—	4	54	75	—
6	Waxy	1*	7*	67	80*	10*	46*	78	75*	0*	2*	68	—
	Cleaned	0*	9*	54	57*	3*	55*	81	67*	3*	33*	92	—
7	Waxy	0*	3*	75	29*	2*	39*	79	39*	0*	14*	69	—
	Cleaned	45*	77*	60	—	16*	42*	57	25*	6*	62*	63	—
9	Waxy	0*	1*	72	73	4*	23*	84	78*	0*	0*	16	—
	Cleaned	0*	2*	51	33*	3*	49*	74	66*	11*	8*	59	—
10	Waxy	—	17*	71*	58*	—	13*	72*	67*	—	4*	24*	59*
	Cleaned	—	1*	17*	32*	—	6*	57*	27*	—	7*	59*	44*
27	Waxy	0	1	0	3	0	5	18	22	0	21	47	28
	Cleaned	0	0	0	0	0	5	17	11	0	10	22	2
84	Waxy	0	0	0	0	0	0	0	0	0	0	0	0

* 100 seeds per sample.

tion the seeds germinated at the low temperatures to such an extent that sample plantings after four months were impracticable. Ten degrees C. seemed to be too high to after-ripen the seeds sufficiently. In every case the germination of the cleaned seeds was superior to that of the waxy.

Figure 1A shows the appearance of seedlings from fresh seeds which had received three months of low temperature treatment prior to planting in the greenhouse. Here the points mentioned above may be noted.

Dry storage for one to seven months increased the germination of seeds after-ripened for three months regardless of the stratification temperature (Table III and Figs. 1B and 1C). This good effect persisted up to nine or

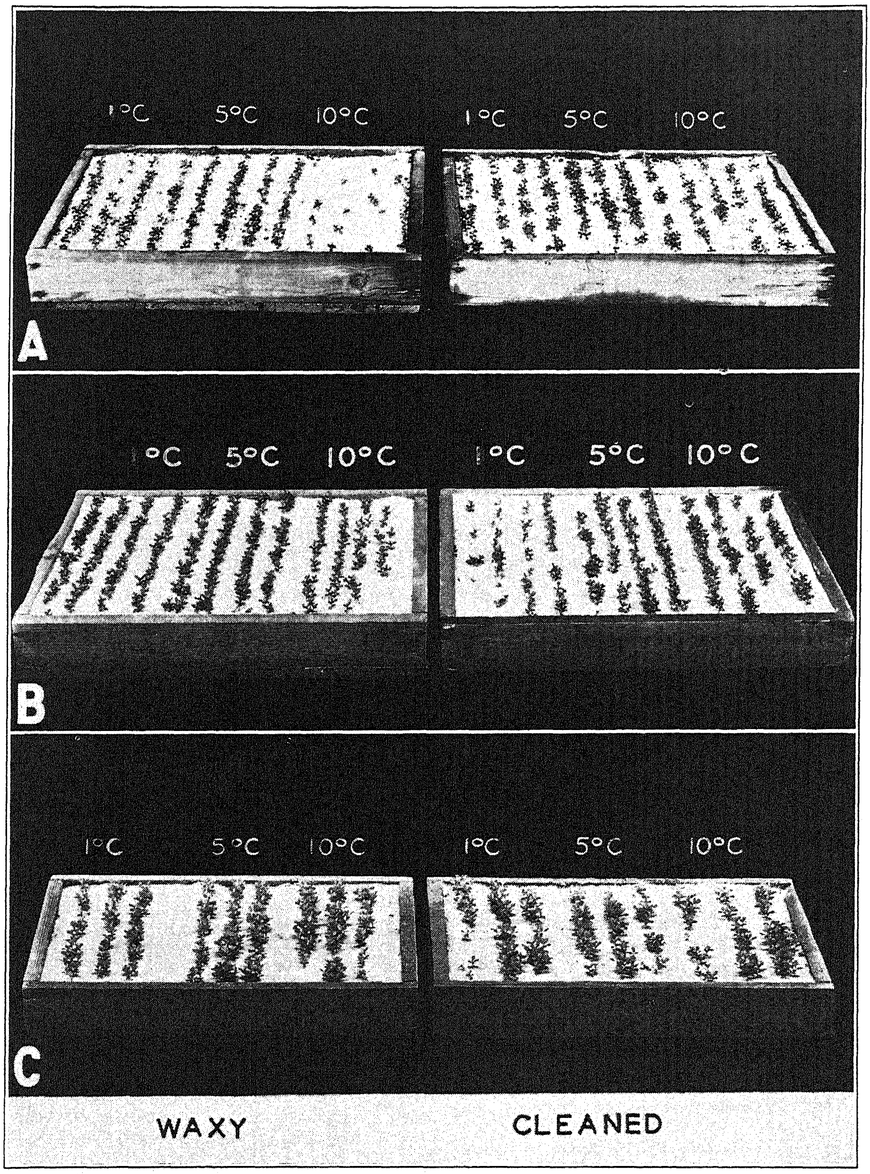

FIGURE 1. A. Fresh seeds; stratified three months; planted in greenhouse December 23, 1930; photographed January 26, 1931. B. Seeds stored dry three months; stratified three months; planted in greenhouse March 24, 1931; photographed April 23, 1931. C. Seeds stored dry seven months; stratified three months; planted in greenhouse July 24, 1931; photographed August 24, 1931.

ten months, but 1° and 5° C. were the only effective stratification temperatures for seeds stored dry for these periods.

Ten degrees C. which proved ineffective for fresh waxy seeds was much more effective for seeds stored dry for one to seven months, even exceeding 1° or 5° C. in the case of cleaned seeds dried for three, four, five, six, or seven months (Table III). After seven months of dry storage, however, there was an abrupt fall in germination percentages from seeds treated in this manner.

Similar behavior was noted for cleaned seeds after-ripened at 10° C. The increased effectiveness of 10° C. as an after-ripening temperature following dry storage is further indicated by results of stratification tests on seeds which had been stored for 27 months (Table III). These seeds were of the 1928 crop reported in the first part of this paper. In this case 10° C. had become the most effective after-ripening temperature and the waxy seeds were superior to the cleaned ones in germinative ability. This latter possibility is also shown in the decreased germination percentages obtained from cleaned seeds after-ripened at 1° or 5° C. after six or seven months of dry storage (Table III).

Tests made on waxy seeds which had been stored dry in a seed locker for seven years revealed a complete loss of vitality.

SUMMARY

1. Dormancy in bayberry seeds may be effectively overcome by low temperature stratification.

2. A period of three months at 5° C. is effective for after-ripening. Fresh seeds thus treated give 78 to 85 per cent germination when planted in the greenhouse.

3. Seeds from which the wax has been removed give better germination when they are fresh but seem to lose vitality more rapidly in dry storage than waxy seeds.

4. Ten degrees C. which is ineffective as an after-ripening temperature for fresh seeds becomes more effective when the seeds have been stored dry for one to ten months and proves better than 1° or 5° C. for after-ripening seeds that have been in dry storage for 27 months.

5. Seedlings may be produced on a commercial scale by planting outside in the fall and mulching or covering with boards over winter. Seeds should not be planted too early as they will germinate and the seedlings will be killed before spring. Three months of cold should be the maximum. If a cold storage room just above freezing were available it would be better to put the seeds at this temperature for three months prior to the advent of warm weather when they could be planted outside. This would eliminate the uncertainty of the temperatures in the cold frame.

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GERMINATION OF SEEDS OF THE SILVER BELL, HALESIA CAROLINA

JOHANNA GIERSBACH AND LELA V. BARTON

INTRODUCTION

Jack[1] states that all seeds with a hard bony covering may commonly be expected to grow in the second rather than in the first year, especially if not planted until spring. Among the plants possessing such seeds he names *Halesia*.

Experiments with seeds of *Halesia carolina* L. were started in this laboratory in an effort to find a method for hastening their germination. Counted numbers of fruits were used in each of the tests. Since some fruits may be entirely empty while others may contain from one to four embryos (Fig. 1), cutting tests of each crop used (except 1929 Lane crop) were made to determine the actual number of good embryos present in two random samples of 100 fruits each. Individual crops were found to vary in embryo

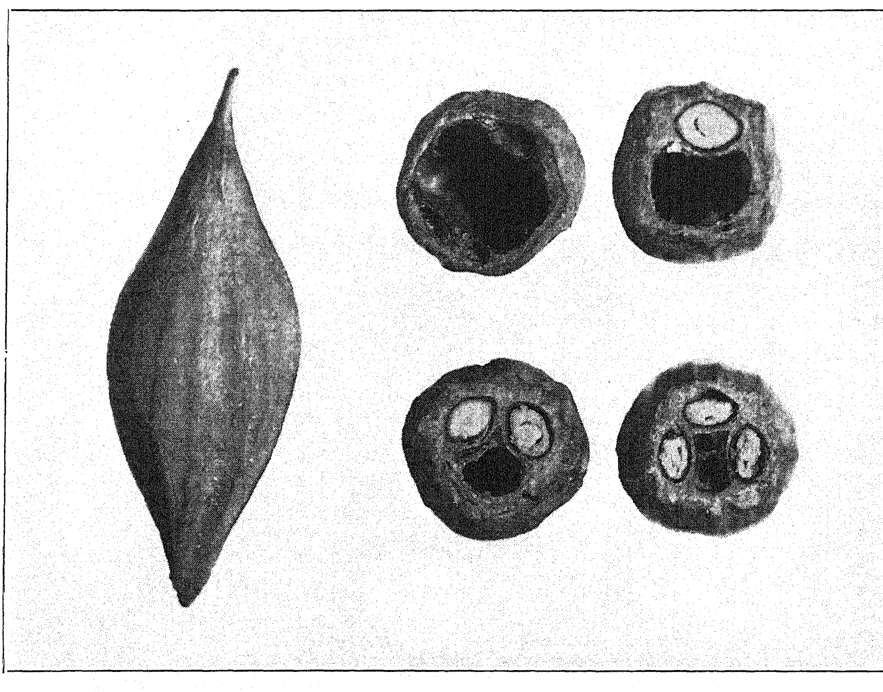

FIGURE 1. *Halesia carolina* L. An entire fruit and four cross sections of fruits showing varying number of embryos. X $3\frac{1}{2}$.

[1] Jack, J. G. Patience with germinating seeds. Garden & Forest **7**: 135–136. 1894.

production. Germination percentages appearing in the tables were calculated on the basis of the number of good embryos present in the 200 fruits used for the cutting tests. Two general methods were used in the germination experiments. One method consisted of plantings made directly in flats which were given various treatments. Other tests were made in constant temperature ovens, from which samples were planted in the greenhouse at various intervals.

Some of the plantings which were made directly in flats were kept permanently in the greenhouse. Others were kept in greenhouses at various temperatures for one, two, or three months and then transferred to cold frames which were left open or covered with boards or mulch during the winter. Still others were planted in flats which were put directly in the cold frames in the fall and allowed to remain there for one or two winters. A mixture of one-fourth sand, one-fourth granulated peat moss, and one-half wood soil was used for flat plantings.

In the case of the oven tests the fruits were mixed with moist granulated peat moss and kept for various lengths of time at constant low temperatures or at high temperatures followed by low. After these periods the fruits were picked out of the peat moss and planted in the greenhouse (kept at 21° C. except in summer) in the soil mixture described above. The peat moss used throughout the experiments was the imported granulated form obtained from Atkins and Durbrow, Inc., 165 John Street, New York City.

RESULTS

PLANTINGS MADE DIRECTLY IN FLATS

Fruits of the 1929 crop obtained from Thomas J. Lane, seedsman, Dresher, Pennsylvania, were planted in flats in November of that year. Some of the flats were kept permanently in greenhouses with temperatures of 10°, 13°, 16°, 18°, and 21° C. except in summer when the temperatures could not be controlled. Others were left in a greenhouse at 18° C. for three months and then transferred to open, mulched, or board-covered cold frames. Still other flats were put immediately into open, mulched, or board-covered frames.

The results of these various tests are shown in Table I. It will be seen that fairly good germination (33.4 to 44.0 per cent) was obtained after one winter in the cold frames when the fruits were first given three months in germination condition in a greenhouse at 18° C. Comparatively few additional seedlings were obtained after the second winter. Final seedling counts after each winter were made in June or July since most of the seedlings appeared in May or June.

On the other hand fruits that had been planted directly in the cold frames germinated poorly after the first winter (Table I) but gave many

TABLE I

SEEDLING PRODUCTION OF 1929 LANE CROP OF HALESIA CAROLINA L. IN COLD FRAMES AND GREENHOUSE. DUPLICATE FLATS OF 700 FRUITS EACH. EXPERIMENT BEGUN NOVEMBER 16, 1929

Treatment		Germination percentage* after			
		One winter		Two winters	
Continuous cold frame	Open	4.0	3.4	32.4	29.4
	Board-covered	3.0	4.2	49.0	52.0
	Mulched	14.0	23.0	71.0	73.0
18° C. for 3 months followed by cold frame	Open	33.4	40.0	35.0	50.0
	Board-covered	38.0	34.0	53.0	52.0
	Mulched	44.0	43.0	64.0	56.0
Continuous greenhouse	10° C.†	1.2	—	5.2	—
	13° C.†	3.2	—	10.4	—
	16° C.†	1.6	—	7.4	—
	18° C.†	3.0	—	5.4	—
	21° C.	4.0	3.0	13.0	8.0

* Based on number of fruits planted.
† Single flats of 500 fruits.

additional seedlings after the second winter outside. Either board-covered or mulched condition proved superior to the open frame for inducing germination. Figure 2 indicates very clearly the advantage gained by planting seeds of this crop at a high temperature for three months before they were exposed to low temperatures outside. The seedling stand of flat A which

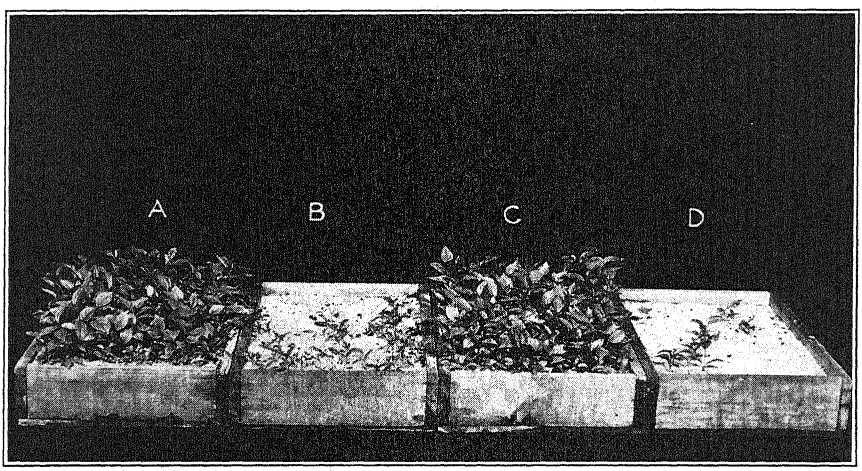

FIGURE 2. *Halesia carolina* L. 1929 crop, Lane. Seedlings produced after one winter in a board-covered cold frame (A) with and (B) without a preceding period of three months in a greenhouse at 18° C., and in a mulched cold frame (C) with and (D) without the preceding treatment.

was kept at 18° C. for three months and then put in a board-covered frame about February 1, far exceeds that of flat B which was put in the board-covered frame when the fruits were first planted in November. Flats C and D show similar plantings in the mulched cold frame. These photographs were taken after one winter outside. It would seem, then, that the effectiveness of a previous high temperature treatment in overcoming dormancy at low temperatures is unquestioned in this case.

Fruits (500 or 700 in each flat) allowed to remain at high greenhouse temperatures throughout two winters produced very few seedlings, thus indicating the necessity of a cold period previous to germination.

Similar outside plantings were made of fruits of the 1930 crop obtained from Lane, cutting tests of which showed 211 good embryos in 200 fruits. In this case only the board-covered cold frame was used and some of the flats were placed at 13°, 18°, 21°, and 27° C. for periods of one, two, and three months before they were transferred to the cold frame. All of the

TABLE II

SEEDLING PRODUCTION OF 1930 CROP (LANE) OF HALESIA CAROLINA L. IN BOARD-COVERED COLD FRAME WITH AND WITHOUT PRECEDING PERIODS AT VARIOUS GREENHOUSE TEMPERATURES. EXPERIMENT BEGUN NOVEMBER 4, 1930

Greenhouse treatment		Per cent germination* in flats†					
Temp. ° C.	Months	a	b	c	d	e	Average
13	1	28	27	20	20	22	23.4
	2	33	37	31	32	30	32.6
	3	45	37	34	34	37	37.4
18	1	21	25	18	28	25	23.4
	2	35	31	31	30	28	31.0
	3	38	54	39	28	28	37.4
21	1	30	30	29	30	40	31.8
	2	38	37	52	47	46	44.0
	3	49	44	43	47	49	46.4
27	1	30	28	28	36	33	31.0
	2	38	34	42	32	41	37.4
	3	47	38	35	48	49	43.4
Continuous cold frame		26	38	34	40	30	33.6

* Based on 106 embryos per 100 fruits as shown by cutting tests.
† 700 fruits per flat per test.

outside plantings including those without previous high temperature treatment gave good germination percentages after one winter (Table II). However, the average seedling production of fruits which were given a previous treatment of two or three months at 21° or 27° C. showed up to 12 per cent increase (Table II) over those without high temperatures. In view of the fact that these averages represent five flats each containing 700 fruits for each condition, these differences are significant. No marked

differences in effects of the high temperatures tried were noted (Fig. 3), although 21° C. seemed to be slightly more favorable. Three months at high temperatures was more effective than one or two months. Seedlings were counted each month from May to October. Most of the seedlings appeared in May and June.

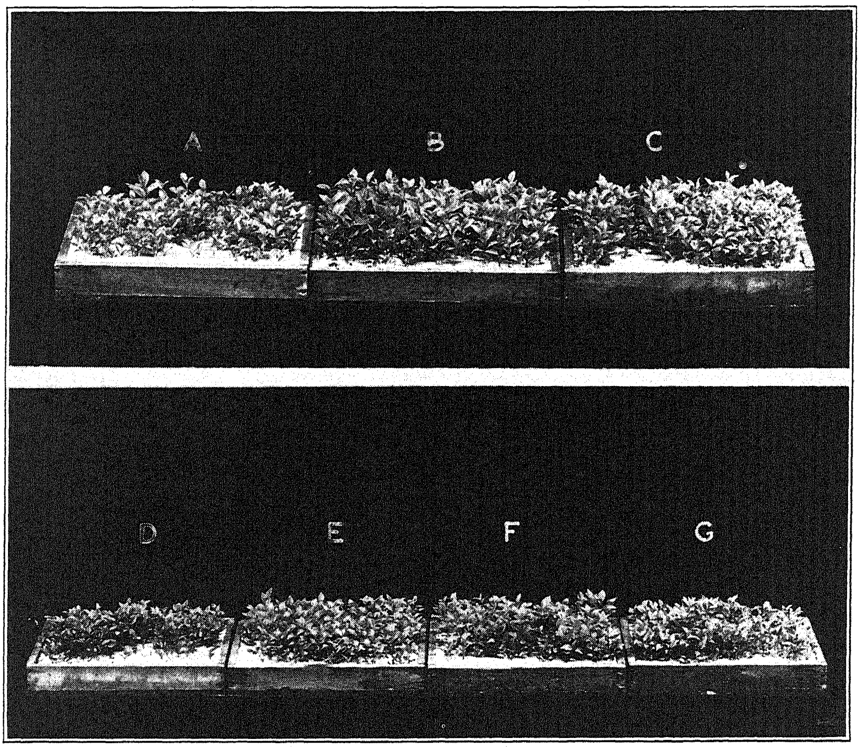

FIGURE 3. *Halesia carolina* L. 1930 crop, Lane. Seedling production after one winter in board-covered cold frame preceded by greenhouse treatment. A. 1 month at 21° C., B. 2 months at 21° C., C. 3 months at 21° C., D. 3 months at 13° C., E. 3 months at 18° C., F. 3 months at 21° C., and G. 3 months at 27° C.

Obviously different crops vary in dormancy since one winter in the cold frames without previous high temperature treatment was not sufficient for good seedling production in the 1929 crop. Green fruits collected in Yonkers in September 1930 and planted immediately showed the effectiveness of a period at a high temperature (21° C. for three months) previous to placing in a mulched or board-covered cold frame (Table III). This is especially marked in the number of seedlings produced up to July 15, 1931. A count of the seedlings on September 15, 1931 showed that the number of seedlings from those flats which had been in the cold frames the entire

TABLE III

SEEDLING PRODUCTION OF HALESIA CAROLINA L. 1930 CROP (YONKERS) IN MULCHED AND BOARD-COVERED COLD FRAMES WITH AND WITHOUT A PRECEDING PERIOD OF THREE MONTHS AT 21° C. EXPERIMENT BEGUN SEPTEMBER 15, 1930.

Maturity of fruits	Greenhouse treatment	Cold Frame	No. of fruits per flat	Per cent germination* in duplicate flats after: 10 months		12 months	
Mature	21° C.	Board-covered	600	60	—	63	—
Green	21° C.	Mulched	500	26	59	27	61
		Board-covered	500	53	53	54	55
Green	None	Mulched	700	11	21	25	35
		Board-covered	700	16	6	32	18

* Calculated on basis of 126 embryos in 100 fruits as shown by cutting tests.

winter had almost doubled while few additional seedlings had appeared in those which had been given the previous high temperature treatment. A similar collection of mature seeds planted in November 1930 gave 63 per cent germination after three months in a greenhouse at 21° C. followed by board-covered cold frame (Table III).

To be of production value seedlings should be produced abundantly in the early spring. A period at high temperature preceding outside planting in a cold frame induced this desired prompt germination in all cases regardless of crop variation.

OVEN TESTS

With the 1930 fruits obtained from Lane and the 1930 green and mature fruits collected in Yonkers, stratifications were made in moist acid peat moss in electrically controlled ovens at constant temperatures of 1°, 5°, and 10° C. Other stratifications made at higher temperatures (15°, 20°, and 25° C.) were transferred after one, two, and three months to the regular low temperature stratification (1° and 5° C.). Samples of fruits (100 each) were removed from stratification after one to seven months at low temperatures and planted in a greenhouse (21° C.) in a mixture of soil, sand, and peat moss. The greenhouse temperature could not, of course, be maintained during the summer months but the additional heat appeared to hasten rather than to interfere with germination.

Table IV and Figure 4 show the results of these tests for the 1930 Lane crop. From the results of the outside plantings of these same seeds reported in Table II, one would expect to find a period of two or three months at 21° or 25° C. prior to low temperature treatment beneficial. This was not shown by the oven tests. In fact the seeds with constant low temperature stratification for five, six, or seven months gave better germination than those treated in any other way. After-ripening proceeded more rapidly at

TABLE IV

SEEDLING PRODUCTION OF HALESIA CAROLINA L., 1930 CROP (LANE), FROM FRUITS STRATIFIED AT HIGH TEMPERATURES FOLLOWED BY LOW, OR AT CONSTANT LOW TEMPERATURES PRIOR TO PLANTING IN THE GREENHOUSE. EXPERIMENT BEGUN NOVEMBER 4, 1930

Stratification at			Per cent germination* in greenhouse after months at low temperature						
High temp.		Low temp. ° C.							
° C.	Months		1	2	3	4	5	6	7
15	1	1	2	1	7	22	22	4	—
		5	2	4	6	11	25	4	—
	2	1	4	25	25	21	14	17	—
		5	4	17	35	16	12	17	—
	3	1	23	21	42	19	11	—	—
		5	10	12	19	12	3	—	—
20	1	1	0	3	9	16	19	9	—
		5	2	5	5	16	20	8	—
	2	1	4	16	25	24	16	5	—
		5	1	18	16	19	9	9	—
	3	1	11	15	23	9	10	—	—
		5	5	7	22	4	5	—	—
25	1	1	2	3	11	20	25	6	—
		5	0	6	6	10	23	0	—
	2	1	10	18	15	26	9	8	—
		5	8	8	5	5	3	0	—
	3	1	4	11	26	13	15	—	—
		5	6	13	22	5	8	—	—
None		1	—	1	6	2	28	42	44
		5	—	4	7	2	24	25	48
		10	—	1	2	1	7	22	25
Control—Dry storage at room temp.			—	8	1	1	2	5	—

* Based on 106 embryos per 100 fruits as shown by cutting tests. 100 fruits per sample.

1° or 5° C. than at 10° C. The low germination (1 to 8 per cent) of the dry seeds, however, definitely proved the necessity for an after-ripening period.

It should be kept in mind that all sample plantings of stratified seeds in the greenhouse were made with lots of 100 fruits each while 500 to 3000 each were used for the outside plantings. This might account for the fact that germination of sample plantings was more variable than for outside plantings of the same seed crop. It is also possible that the fluctuating outside temperatures were more favorable for after-ripening than the constant low temperatures of the ovens. The board-covered frame afforded some protection for the flats, but it undoubtedly allowed freezing and thawing to occur. The mulched frame on the other hand maintained a

temperature which fluctuated between narrow limits but remained above freezing.

The rapidity of germination in the greenhouse varied with the individual crops and with the stratification period. Seedlings from well after-ripened fruits began to appear in 30 to 45 days after samples were planted in the greenhouse while 60 days were required for the earliest germination of fruits with shorter stratification periods. In the latter case, germination extended over a period of about 150 days. This period became shorter

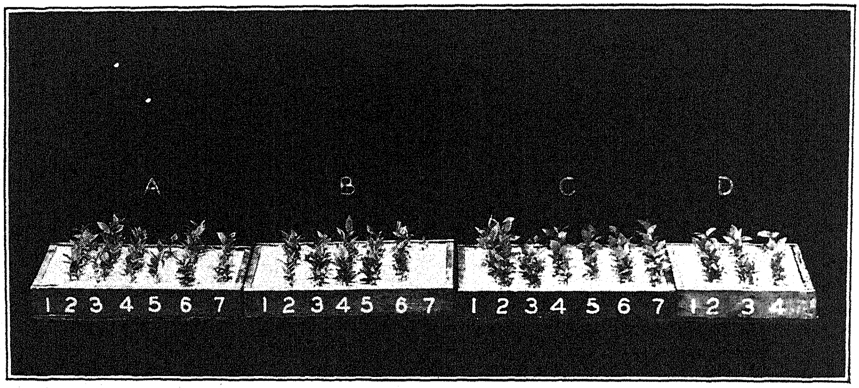

FIGURE 4. *Halesia carolina* L. 1930 crop, Lane. Seedling production from seeds planted in the greenhouse after A. 1 month at high temperature + 5 months at low temperature, B. 2 months at high temperature + 4 months at low temperature, C. 3 months at high temperature + 3 months at low temperature, and D. 6 months at low temperature. In A, B, and C, (1) dry fruits planted as controls, (2) 15°+1° C., (3) 15°+5° C., (4) 20°+1° C., (5) 20° +5° C., (6) 25°+1° C., and (7) 25°+5° C. In D, (1) dry fruits planted as controls, (2) 1° C., (3) 5° C., and (4) 10° C.

with increasing length of time in the after-ripening conditions. Fruits with a previous stratification period of six months completed their germination in about 40 days.

It is interesting to note that dry, untreated seeds planted in the greenhouse in January, February, March, April, and May as controls for the stratified seeds showed no germinations until June or July when each of the plantings started to germinate. This may be explained by the fact that as the season advanced it became impossible to control the temperature in the greenhouse. Weather records for May and June show great variations in the temperatures in the sunlight (from 16° to 57° C.). It is possible that widely alternating temperatures will bring about germination without previous low temperature treatment. As yet, this has not been tested. Narrowly alternating temperatures as daily 10° to 20° C. do not induce germination.

Oven tests made with mature fruits collected in Yonkers in 1930

showed very little differences in effectiveness of the different stratification methods tried (Table V).

Oven stratification of the green fruits (Table VI) confirm the data obtained from outside plantings of these same seeds (Table III).

The main advantage to be gained by using a period of high temperature stratification preceding the low temperature treatment for green

TABLE V

SEEDLING PRODUCTION OF HALESIA CAROLINA L. 1930 CROP MATURE (YONKERS) FROM FRUITS STRATIFIED AT HIGH TEMPERATURES FOLLOWED BY LOW, OR AT CONSTANT LOW TEMPERATURES PRIOR TO PLANTING IN THE GREENHOUSE. EXPERIMENT BEGUN NOVEMBER 15, 1930.

Stratification at			Per cent germination* in greenhouse after months at low temperature						
High temp.		Low temp. ° C.							
° C.	Months		1	2	3	4	5	6	7
15	1	1	71	33	62	51	65	14	35
		5	46	29	63	51	71	21	35
	2	1	56	67	35	62	52	44	8
		5	38	48	35	52	41	40	14
	3	1	75	40	63	17	33	27	—
		5	67	22	60	19	43	46	—
20	1	1	29	19	52	51	76	27	37
		5	33	40	75	51	57	16	32
	2	1	44	51	48	52	49	21	14
		5	51	49	43	51	52	41	12
	3	1	43	17	81	43	37	30	—
		5	76	24	68	41	32	32	—
25	1	1	24	51	63	13	65	37	17
		5	29	30	41	57	41	30	20
	2	1	35	44	54	60	48	33	8
		5	48	65	38	52	17	25	12
	3	1	67	17	83	37	33	24	—
		5	57	29	57	33	29	29	—
None		1	—	21	30	70	67	42	42
		5	—	37	35	60	59	50	24
Control—Dry storage at room temp.			—	16	13	16	28	36	9

* Based on 126 embryos per 100 fruits as shown by cutting tests. 50 fruits each sample except samples from constant low temperatures and dry control where 100 fruits each were used.

seeds is in producing a more prompt stand of seedlings in the greenhouse. The best germination percentage (84 per cent) was obtained from seeds which had been at 15° C. for two months after which they were transferred to 5° C. for two months. On the other hand seeds stratified at a constant

temperature of 5° C. for three months gave 62 per cent seedling production when planted in greenhouse flats. Germination in the former case was complete in 53 days and in the latter case 78 days were required to complete germination.

TABLE VI

SEEDLING PRODUCTION OF HALESIA CAROLINA L. 1930 CROP GREEN (YONKERS) FROM FRUITS STRATIFIED AT HIGH TEMPERATURES FOLLOWED BY LOW, OR AT CONSTANT LOW TEMPERATURES PRIOR TO PLANTING IN THE GREENHOUSE. EXPERIMENT BEGUN SEPTEMBER 15, 1930.

Stratification at			Per cent germination* in greenhouse after months at low temperature						
High temp.		Low temp. ° C.							
° C.	Months		1	2	3	4	5	6	7
15	1	1	—	60	60	5	21	12	40
		5	—	44	37	—	11	25	19
	2	1	5	44	51	24	37	38	21
		5	11	84	46	27	38	41	27
	3	1	32	3	19	16	30	30	10
		5	35	2	40	33	51	25	11
20	1	1	—	67	40	2	29	19	27
		5	—	8	29	11	29	19	17
	2	1	6	40	46	46	22	56	10
		5	10	51	37	43	46	48	21
	3	1	38	2	29	33	40	22	16
		5	57	19	48	29	54	33	25
25	1	1	—	56	62	37	11	24	24
		5	—	2	52	22	37	32	35
	2	1	5	67	43	51	33	40	16
		5	8	51	0	14	37	48	14
	3	1	51	10	48	35	32	41	29
		5	32	3	40	35	46	52	24
None		1	—	—	45	39	9	18	14
		5	—	—	62	36	3	29	10
Control—Dry storage at room temp.			—	—	14	17	3	11	6

* Based on 126 embryos per 100 fruits as shown by cutting tests. 50 fruits each sample except samples from constant low temperatures and dry control where 100 fruits each were used.

It was apparent throughout the germination tests made on fruits which had been in constant temperature ovens that there was an optimum time for stratification (Tables IV, V, and VI). Although the exact time required for after-ripening varied with the different crops, too long a period in stratification always resulted in poor seedling production in the greenhouse. There was practically no germination in the ovens.

SUMMARY

1. Seeds of individual crops of *Halesia carolina* vary in dormancy but all seeds responded to low temperature stratification.

2. A high temperature (13° to 27° C.) period of one to three months preceding planting in mulched or board-covered cold frames improved the germination markedly in two cases and significantly in a third.

3. Good germination was obtained the first year by this method regardless of the crop.

4. Greenhouse plantings of seeds which had been stratified in ovens at constant low temperatures (1°, 5°, or 10° C.) and at high temperatures (15°, 20°, or 25° C.) followed by low (1° or 5° C.) in general confirmed the results of the outside plantings.

5. In order to obtain a good stand of seedlings the first spring after planting, nurserymen should plant the fruits in flats in October or November. These flats should be kept in a greenhouse of 21° to 27° C. until January when they should be transferred to a mulched or board-covered cold frame.

GERMINATION AND STORAGE OF WILD PLUM SEEDS

JOHANNA GIERSBACH AND WILLIAM CROCKER

INTRODUCTION

Wild plum (*Prunus americana* Marsh.) is sometimes used as understock for plums (3, v. 3, p. 2827) where great frost hardiness is required. It is also used as a parent in breeding plums (1, 8) in cold regions of North America because of its hardiness. A letter from a nurseryman in Colorado stated that wild plum is inclined to heavy fruiting certain years and to light fruiting other years and asked whether the seeds could be carried over from fruitful years to be used for seedling production in less fruitful years. With many trees periodic production of seeds occurs. In the red pine (*Pinus resinosa* Ait.) several years may elapse between good seed crops. Because of the existence of periodic bearing, the best methods of storing tree seeds to maintain their vitality is of considerable importance to nurserymen. Any information on the storage, after-ripening, and germination of rosaceous seeds is of interest to nurserymen because of the great importance of this family of plants in ornamental planting and fruit production.

LITERATURE

A recent paper by Crocker and Barton (5) reviewed the literature on the after-ripening and germination of rosaceous seeds as well as the retention of vitality by these seeds under various storage conditions. They pointed out the facts that seeds of this family require a considerable period in a low temperature to after-ripen or prepare them for germination and that the after-ripening involves changes in dormant embryos that must take place before the seeds can germinate. They found that apple seeds fall in vitality rather slowly with dry storage and that two and one-half year old seeds gave a perfect stand of seedlings if the seeds were properly after-ripened before planting. They also found that *Rosa* seeds of various species degenerated relatively slowly in dry storage, retaining considerable vitality after two or three years of dry storage.

In a recent article, Flemion (7) reported a thorough study of the after-ripening and germination of European mountain ash (*Sorbus aucuparia* L.) seeds. They after-ripened in two to four months when stratified at 1° C., or at daily or weekly alternating temperatures of 1° to 5° C. Other stratification temperatures were less favorable. With two years of dry storage under a considerable range of humidity and temperature, including room temperature in both sealed and open vessels, the seeds showed no fall in vitality. High humidities or very low humidities proved unfavorable for

storage at room temperatures, but less unfavorable at low temperatures. Twenty-five degrees C. constant proved very unfavorable for storage. Flemion showed that the embryos were dormant, also that the seed coats played a considerable rôle in the dormancy of the seeds. The embryos were fully after-ripened in intact, properly stratified seeds many days before the intact seeds were capable of germination. After-ripened embryos absorbed and permitted water to move through them with much greater freedom than unafter-ripened embryos. Change in water relations of the embryos was considered to be of fundamental importance in after-ripening in stratification. She also made a study of enzyme changes during after-ripening and germination. Catalase rose continuously with after-ripening and germination but the most favorable temperatures for after-ripening did not prove to be the most favorable for rise in catalase activity, so there was no strict relation between catalase activity and after-ripening. Peroxidase activity rose with after-ripening of the seed but fell again as germination progressed. Emulsin and amylase showed no change with after-ripening and germination.

EXPERIMENTAL RESULTS

Effect of conditions on after-ripening and germination. Table I shows the effect of various temperatures, the pericarp, and acidity of the substratum upon the after-ripening and germination of wild plum seeds. These seeds were of the crop of 1926 and were shipped from Andrew's Nurseries, Boulder, Colorado, in moist sand immediately after they were collected and cleaned, so the seeds were never dried. The experiment was started on September 28, 1926. An examination of the seeds showed that all contained good embryos. Two hundred seeds were used for each experiment. The seeds were mixed with moist peat (pH about 4) or with moist peat neutralized with precipitated lime carbonate, placed in large-mouthed bottles with a cheesecloth cover, and put into the electrically regulated chambers. The cultures were examined at regular periods, germinated seeds counted, decayed ones removed, and the water brought up to the proper content.

Examination of Table I shows that 5° C. was most favorable for the after-ripening and germination of these seeds, giving 71.5 per cent after seven months; 1° C. is next with 52.5 per cent after the same period; and 10° C. far less favorable, giving 22 per cent. Weekly alternation between −15° and 5° C. proved very unfavorable, giving only 7.5 per cent germination and leading to the decay of all the non-germinating seeds. This furnishes further evidence against the erroneous theory that seeds with dormant embryos are aided in their after-ripening by freezing. The seeds with the pericarp removed (5° C.) after-ripened and germinated more promptly than those with pericarp intact (5° C.), but so many decayed

TABLE I

EFFECT OF TEMPERATURE, ACIDITY, AND PERICARP UPON THE AFTER-RIPENING AND GERMINATION OF WILD PLUM SEEDS
Seeds not allowed to dry before put into germinator. Experiment started September 28, 1926; all seeds contained good embryos; sterilized with 0.25 per cent Uspulun for 0.5 hours; 200 seeds used in each condition; 1926 crop from Boulder, Colorado

Temperature	Per cent germination after										Per cent non-germinated seeds decayed at close of experiment	
	4 months		5 months		$5\frac{1}{2}$ months		6 months		7 months			
	Peat	Neutral peat	Peat	Neutral peat	Peat	Neutral peat	Peat	Neutral peat	Peat	Neutral peat	Peat	Neutral peat
1° C.	0	0	0	0	38	37	38	52	44	53	80	50
5° C.	0	0	0	54	57	71	60	71	61	72	50	66
10° C.	2	0	10	0	16	12	18	16	23	22	65	20
Weekly alternation between −15° and 5° C.	0	—	8	—	8	—	8	—	8	—	100	—
5° C. pericarp removed	4	—	26	—	49	—	49	—	49	—	100	—

in the germinating media that the final yield was only 49 per cent. At 1° C. and at 5° C. there was somewhat higher germination in neutral than in acid peat. The reverse was true at 10° C. It is a question whether there was any advantage in neutralizing the peat. There was somewhat more decay of non-germinating seeds in the peat than in the neutral peat, but this difference was not constant and may not be significant.

The speed of after-ripening at various low temperatures, independently of germinations at these temperatures, was studied by the following procedure. The seeds were removed from the low temperatures at different times before germination began or was extensive at the low temperatures. Table II gives data on this subject. The seeds that were sown without

TABLE II

AFTER-RIPENING OF WILD PLUM SEEDS AT LOW TEMPERATURES

Seeds stored dry in laboratory 39 months, then stratified in peat at 1°, 5°, and 10° C. Samples removed after 3, 4, and 5 months and planted in flats in greenhouse; seedlings counted 17 days after planting; 100 seeds used in each test; 1926 crop from Boulder, Colorado

Stratification temperature	Period of stratification and per cent seedling production in greenhouse after stratification		
	3 months	4 months	5 months
1° C.	2	23	39
5° C.	6	15	52
10° C.	0	0	8
Check sown dry	0	0	0

previous stratification gave no seedling production. Stratification at 5° C. for three months gave 6 per cent seedling production; for four months, 15 per cent; and for five months, 52 per cent. Stratification at 1° C. gave 2 per cent seedling production after three months; 23 per cent after four months; and 39 per cent after five months. Stratification at 10° C. gave no seedling production after three or four months' stratification, and only 8 per cent after five months' stratification. Dry stored seeds planted directly in the greenhouse gave no seedlings. Five degrees C. was a favorable temperature for after-ripening of these seeds, although 1° C. was nearly as good. Flemion (7) found 1° C. very favorable for the stratification of European mountain ash seeds. In practice it is likely that good seedling production of wild plum can be obtained by stratifying the seeds at about 5° C. in moist granulated peat for five months just previous to early spring planting. The lower temperatures of the outside beds may lead to more seeds after-ripening and a still higher seedling production.

Figure 1 shows the seedling production from the experiment described in Table II.

Seeds of wild plum were planted about one inch deep in flats in the fall and the flats placed in cold frames. One of the cold frames was left

without any cover, another was provided with a tight board roof, and a third was provided with a mulch of leaves four to five inches deep and in addition covered with a tight board cover. The seeds in the open frame had a fluctuation in temperature corresponding to the fluctuation of outdoor soil at one inch depth; the seeds in the board-covered frames experienced less fluctuation in temperature but froze and thawed several

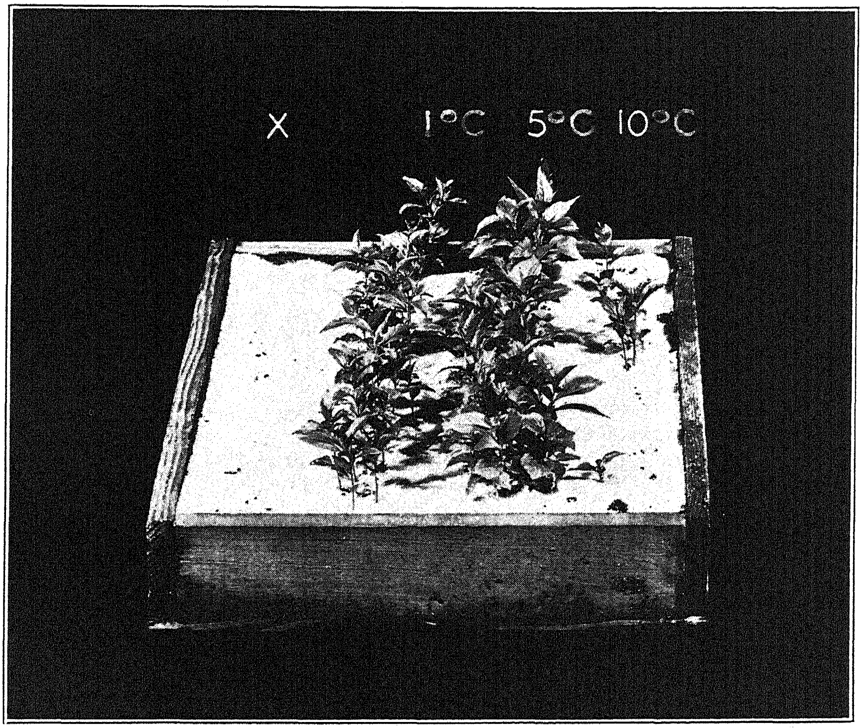

FIGURE 1. Seedling production in the greenhouse 17 days after planting of seeds of wild plum stored dry 39 months before beginning of experiments. X, seeds stored dry until planted, 44 months; 1° C., seeds stratified 5 months in moist peat at 1° C. previous to planting; 5° C., similarly stratified at 5° C. before planting; 10° C., similarly stratified at 10° C. before planting.

times during the winter; while the seeds in the mulched and board-covered frames fluctuated little in temperature and were always held somewhat above freezing.

Table III shows the effects of these treatments on seeds of the 1926 Boulder, Colorado crop. In the September 15 planting only 4 per cent of seedlings resulted from planting in the mulched frames while 30.5 per cent of seedlings appeared in the open frames and 46.5 per cent in board-covered

TABLE III

FALL PLANTING OF WILD PLUM SEEDS

Planted in flats and placed in cold frames in the fall: one frame left open, one frame with board roof, and one frame mulched with 4 to 5 inches of leaves at time of planting and covered with board roof; 200 seeds used in each test; seedlings counted July 10, 1927; 1926 crop from Boulder, Colorado

Condition of frame	Per cent seedlings	
	Planted September 15, 1926	Planted November 24, 1926
Open	31	4*
Board cover	47	39
Mulched	4**	57

* Embryo test of 50 seeds on July 10, 1927 showed 40% just ready to germinate, 38% rotted, and 22% with healthy looking embryos.

** Embryo test of 50 seeds on July 10, 1927 showed 50% with empty coats and 50% with rotted embryos.

frames. The poor results from the mulched frames were probably due to the period being so long that many of the seeds germinated and the resulting seedlings were killed under the mulch. In the November planting 56.5 per cent seedlings were produced in the mulched frame, 3.5 per cent in the open frame, and 39 per cent in the board-covered frame. This period was more nearly optimum for the mulched frame with its well regulated temperature. The open frame gave a relatively short period of good after-ripening temperature and the board-covered frame stood intermediate.

TABLE IV

FALL PLANTING OF WILD PLUM SEEDS

Planted in flats in cold frames; 200 seeds used in each test; seedlings counted July 10, 1927; 1926 crop from a seedsman in Pennsylvania

Condition of frame	Per cent seedlings	
	Planted October 26, 1926	Planted September 28, 1926
Open	22	39
Board cover	70	48
Mulched	2*	0**

* Examination of 50 seeds on July 10, 1927 showed 72% of pits empty.

** Embryo test of 50 seeds on July 10, 1927 showed 29% rotten inside hard coat and 71% of seeds with only coats left.

Table IV shows a similar experiment for seeds from another source. In this experiment both September 28 and October 26 proved to be too early planting dates for mulched frames. October 26 proved to be a very favorable planting date for the board-covered frame with 70 per cent seedling production, while September 28 was less favorable with 47.5 per cent seedling production. Both planting dates gave less favorable results

for the open frames than either date for the board-covered frame but better results than either date for the mulched frame.

The successful production of seedlings in a cold frame depends on maintaining a proper after-ripening temperature for just a sufficiently long period to have the seeds ready to germinate with the opening of spring. The effectiveness of an open or board-covered frame will vary with the climate of different regions and with the climate of a given region from year to year. By proper mulching and proper date of fall planting it is likely that one can get good seedling production in any of the colder regions. It is a question, however, whether proper stratification followed by spring planting is not the most effective method for seedling production.

At the same time one of the plantings was made in cold frames, namely September 28, 1926, plantings were made in flats which were held in greenhouses regulated at 10°, 15°, and 23° C. whenever solar insolation or general outdoor temperature did not run the temperatures in the greenhouses above these points. By April 15, 1926 the 10° C. house gave 7 per cent seedling production and the 15° and 23° C. greenhouses gave no seedlings. The last two temperatures were too high to give any after-ripening and germination while the 10° C. house was only slightly effective because of the frequent rise of temperature above 10° C. All seeds not germinated on April 15 were found to be in good condition. The seeds kept well in soil at higher temperatures though such temperatures did not permit of after-ripening and germination.

Effect of sterilization of seeds. In some of the experiments the seeds were sterilized with Uspulun and in others the seeds were not sterilized. Table V shows the effects of sterilizing. All the seeds were dry stored for 26 months before the germination was started. There were four conditions of storage. (1) A set was stored in a seed cabinet which had the temperature of the laboratory and was protected against dust accumulation. (2) Another set was stored on top of the same seed cabinet, had a more variable humidity, and was open to dust accumulation. (3) A third set was stored in a cabinet against the chimney and was at a much higher temperature, above 30° C. They were also more thoroughly dried as was shown by the splitting of most of the pericarps. (4) A fourth set of seeds was stored in the ante-room to the refrigeration room and had a temperature ranging between 7° and 10° C. and a high humidity. With the seeds stored in the seed cabinet, sterilization had little effect; also this period and condition of storage improved the vitality of the seeds, for here the germination percentage was the highest on the average obtained for any set of tests. Sterilization was beneficial for the seeds stored on top of the cabinet. It probably destroyed the superficial spores that accumulated with the dust during the storage. The seeds in the cabinet against the chimney were not benefitted by sterilization but perhaps somewhat injured. The splitting

TABLE V

EFFECT OF STERILIZATION WITH USPULUN ON WILD PLUM SEEDS

Seeds treated with 0.25 per cent Uspulun for 0.5 hours; planted in moist peat at 5° C. Experiment started November, 1928; seeds stored dry 26 months in different conditions before planting; 50 seeds used in each test; 1926 crop from Boulder, Colorado

Temp.	Storage condition															
	Laboratory temperature protected from dust				Laboratory temperature exposed to dust				At 30° C. and above, excessive drying				Moist room 7° to 10° C.			
	Sterilized		Not sterilized		Sterilized		Not sterilized		Sterilized		Not sterilized		Sterilized		Not sterilized	
	% germ.	After days	% germ.	After days	% germ.	After days	% germ.	After days	% germ.	After days	% germ.	After days	% germ.	After days	% germ.	After days
1° C.	88	202	88	202	76	202	46	202	42	342	88	342	40	202	14	202
5° C.	100	253	98	253	98	253	62	253	54	253	58	253	66	253	62	253
10° C.	90	320	86	320	76	320	66	320	68	342	60	342	88	320	62	320

of the pericarps and perhaps seed coats, due to extreme drying, may have allowed the fungicide to come in contact with the embryos and injure them. On the whole, the results were little modified by sterilizing properly stored seeds.

Effect of temporary artificial drying. Temporary artificial drying improves the germination of some cereal seeds (2). It was thought worth

TABLE VI

EFFECT OF TEMPORARY ARTIFICIAL DRYING OF WILD PLUM SEEDS

One hundred seeds per sample for germination at low temperature; 40 seeds per sample for greenhouse planting after 5 months of after-ripening; 1926 crops from Boulder, Colorado

Stored 19 days over	Germination temperature °C.	Per cent germination after months at low temperature						Per cent germination of sample plantings in the greenhouse after 5 months at low temperature
		4	5	6	7	8	9	
Quick lime	1	0	0	10	10	10	10	22
	5	0	6	12	28	30	30	28
	10	0	0	0	0	0	5	0
Conc. H_2SO_4	1	0	0	18	29	29	29	40
	5	0	0	2	25	27	27	22
	10	0	0	2	23	23	15	0
75% H_2SO_4	1	4	4	36	39	39	39	33
	5	4	8	16	21	21	21	16
	10	0	0	0	0	0	3	0
50% H_2SO_4	1	4	17	17	20	20	20	28
	5	0	6	6	37	48	48	38
	10	0	0	2	5	5	5	6
25% H_2SO_4	1	4	36	36	39	39	39	28
	5	0	0	17	44	44	44	40
	10	0	0	0	0	0	0	0
Checks	1	0	0	2	33	40	40	55
	5	0	0	4	61	62	63	50
	10	2	10	12	15	18	21	18
Checks (Planted without after-ripening)								0

while to try it on rosaceous seeds. Table VI shows the effect of 19 days' drying of wild plum seeds over various reagents just previous to sowing them in peat at three different temperatures, 1°, 5°, and 10° C. The table shows that the checks gave better germination at all temperatures than any of the batches dried artificially. In general, too, as in previous experiments, 5° C. was the best of the three temperatures tried for the after-ripening and germination of the seeds. There are two exceptions to this in favor of 1° C. Some such exceptions have appeared in experiments previously reported in this paper, indicating that 1° C. is a close second to

5° C. After five months in the low temperature germinators samples of 40 seeds each were taken from each germination condition and planted in flats in the greenhouse. With five months of after-ripening, 1° and 5° C. were in all cases far superior to 10° C., though there is relatively little difference between 1° and 5° C. In these sample plantings of low temperature after-ripened seeds, the seeds that were not artificially dried gave greater germination than the artificially dried seeds. Wild plum seeds are not improved in their after-ripening and germination by temporary artificial drying.

Retention of vitality in dry storage. As has been shown by various workers (4, 6, 9, 10), seeds that will withstand thorough air drying or more excessive drying are improved in their retention of vitality by lowering the water content to or below a certain critical point, by lowering the temperature of storage and by hermetically sealing if it is accompanied by sufficient drying. These researches also show that the injurious effect of high moisture can be partly overcome by lowering the storage temperature to a point just above freezing.

Table VII shows the effect of dry storage of wild plum seeds in the five conditions mentioned under the heading "Effect of sterilization of seeds" upon the retention of vitality. After various periods of storage in the five conditions the seeds were mixed with moist peat and placed in an oven at 5° C. and allowed to run until germination ceased. In the columns under "Days" in each case are recorded the number of days required for termination of germination. As the table shows, fresh seeds gave 71 per cent germination in 180 days. After 11 months of storage seeds in the seed cabinet gave 58 per cent germination in 240 days; those on top of the seed cabinet gave 84 per cent after 250 days; and those in the ante-room to the refrigeration room gave 68 per cent after 240 days. The other storage periods tested were 18, 26, 30, 42, 46, and 53 months at which time the supply of seeds was exhausted. The table shows that the cabinet against the chimney, which had a temperature of 30° C. or above and which gave excessive drying, was the poorest storage condition used for retention of vitality for a long period. In these the vitality began to fall noticeably after 30 months of storage. After 42 months of storage 8 per cent germinated; after 46 months, 6 per cent; and after 53 months, none germinated. Even after 46 months of storage the other three conditions gave more than 40 per cent germination. After 53 months of storage the seeds kept in the ante-room, in spite of its high humidity, still gave 45 per cent germination in 181 days. The other two favorable temperatures fell considerably below this percentage.

It is unfortunate that these storage experiments did not include seeds that were in sealed storage and kept at various temperatures above and below freezing. From data mentioned in this section and from previous

TABLE VII

EFFECT OF STORAGE UPON THE VITALITY OF WILD PLUM SEEDS

Germination in peat at 5° C. Columns marked days indicate number of days in germinator when germination ceased; 100 seeds used in each test; 1926 crop from Boulder, Colorado

Storage condition	Fresh seeds		Storage time in months													
			11		18		26		30		42		46		53	
	Days	% germ.	Days	% germ.	Days	% germ.	Days	% germ.	Days	% germ.	Days	% germ.	Days	% germ.	Days	% germ.
Fresh seeds	180	71	—	—	—	—	—	—	—	—	—	—	—	—	—	—
Laboratory temperature, protected from dust	—	—	240	58	210	72	253	98	209	94	174	36	235	48	181	16
Laboratory temperature, exposed to dust	—	—	250	84	210	58	253	62	209	62	174	24	235	41	181	4
At 30° C. and above, excessive drying	—	—	—	—	210	62	253	58	209	50	174	8	192	6	181	0
Moist room 7° to 10° C.	—	—	240	68	189	70	253	62	184	66	174	42	235	51	181	45

researches mentioned above it is probable that wild plum seeds with optimum water content in sealed storage and held at the best temperature will retain their vitality for many years. Seeds in dry open storage in a laboratory showed little degeneration in nearly four years. In this condition, moreover, they improved in germination capacity up to 26 or 30 months. It is certain that no special precautions need to be taken to carry these seeds two years in dry storage.

SUMMARY

1. Wild plum seeds require a period of low temperature stratification or a period in a low temperature germinator for after-ripening preparatory to germination. Of the three low constant temperatures tried, 5° C. was the best, 1° C. nearly as good, and 10° C. much less effective. At 5° C. in moist peat more than 50 per cent of the seeds after-ripened in five months as was shown by sample plantings in the greenhouse after various periods of stratification.

2. Seeds planted in flats in greenhouses at 15° and 23° C. as much of the time as outside weather would permit from September to April gave no germination.

Similar treatment in a greenhouse with the minimum of 10° C. gave 7 per cent germination.

3. The seeds not only after-ripened in germinators at 1°, 5°, and 10° C., but they germinated at these temperatures if kept a sufficient period. Five degrees C. proved to be the best of these temperatures for after-ripening and germination, 1° C. next, and 10° C. poorest. Weekly intermittent temperatures at −15° C. and 5° C. gave 7.5 per cent germination but all the non-germinating seeds rotted. Seeds with the pericarps removed after-ripened and germinated more quickly at low temperatures than those with pericarps intact, but the lack of the protection of the pericarp led to the decay of about half of the seeds.

4. Sterilizing the seeds with Uspulun did not prove of advantage, since the seeds are rather resistant to fungal attack in the soil under proper after-ripening and germination conditions.

5. Temporary drying over quick lime and concentrated sulphuric acid reduced germination.

6. Seeds germinated somewhat better and rotted somewhat less in neutralized peat than in acid peat (about pH 4) but the difference was not great and was sometimes in favor of the acid peat.

7. The seeds stored in a laboratory cupboard improved in their germination up to 26 to 30 months and retained more than half their vitality up to 46 months. Seeds stored at 7° to 10° C. showed good germinating capacity after 53 months of storage. Sealed storage and lower temperatures were not tried.

8. Good seedling production was obtained by sowing the seeds in cold

frames in late November and mulching so as to hold the germination bed just above freezing until ready to uncover in April. Earlier planting in mulched beds led to germination under the mulch and loss of seedlings. September planting in a board-covered frame gave 47.5 per cent seedling production and similar planting in an open frame gave 38.5 per cent seedlings.

9. The best way to produce seedlings is to stratify the seeds at about 5° C. for five months just previous to early spring planting in cold frames or sheltered beds.

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DIRECT VERSUS INDIRECT EFFECTS UPON POTATO AMYLASE BY CHEMICALS WHICH INDUCE SPROUTING OF DORMANT TUBERS[1]

F. E. DENNY

INTRODUCTION

When chemical treatments induce growth in dormant buds such as freshly-harvested potato tubers, the suggestion has been made frequently that the effectiveness of the treatment is related to its capacity to hasten the activity of the enzyme systems in the tissue.

We should recognize, however, that such an increase in the enzyme activity could have been brought about in at least two different ways:(a) by a *direct* action of the chemical in activating the enzymes already existing in the tissue, (b) by an *indirect* action upon the living matter, inducing the formation of a greater amount of (or more active) enzymes.

In a previous report (4) on the effect of chemical treatments upon the enzyme activity of potato tubers, it was shown that the increases in the activity of the various enzymes studied were brought about probably by an *indirect* effect, since the observed increases could not be obtained by adding the chemical directly to the expressed juices; only by treating the living and active *tissue* with the chemical and testing the juices subsequently were increases in enzyme activity found.

In the present experiments special attention was given to amylase activity in relation to chemical treatments for breaking dormancy of potato tubers (*Solanum tuberosum* L.). The chemicals used were: ethylene chlorhydrin ($ClCH_2CH_2OH$), sodium and potassium thiocyanate (NaSCN and KSCN), potassium cyanide (KCN), potassium nitrate (KNO_3), and potassium chloride (KCl). The direct and indirect effects of these chemicals upon amylase activity, and the relation of amylase activity to sprouting response, were shown by the following measurements: (a) amylase activity when the chemical was added directly to the expressed juice; (b) amylase activity when the potato tissue was treated with the chemical and juices were expressed from the treated tissue at a subsequent period before sprouting occurred; (c) the sprouting response of the potatoes to the chemical treatments.

The results corroborate the view that the effect of the chemical treatment upon amylase activity must be indirect; for example, ethylene chlorhydrin which has very little effect upon potato amylase when added to potato *juice*, greatly increases the amylase if the *tissue* is treated with the chemical; potassium cyanide hastens the activity if the chemical is added

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to the *juice* but has little effect upon amylase if the *tissue* is treated with it; and sodium thiocyanate which has a retarding effect when the chemical is added to the *juice* does not retard but may even increase the activity if the *tissue* is treated.

Nor were high amylase activities of treated tissues correlated closely with favorable sprouting responses. It is true that ethylene chlorhydrin induced high amylase and good sprouting, but sodium thiocyanate brought about successful sprouting even when potato juices from such treatments did not show amylase activities that were materially greater than juices from untreated tubers.

METHODS

Chemical treatments. The tubers were cut into pieces weighing approximately 28 grams each. For the ethylene chlorhydrin treatments the dip method was used; the pieces were placed in wide-mouthed fruit jars, were covered with a solution the strength of which is shown in the appropriate column of each table; this solution was then poured off at once and the excess liquid dislodged by shaking; the jar was closed, and was stored at a temperature of approximately 20° C. for 24 hours. The treated seed-pieces were planted at once after removal from the jars in soil in flats.

For the treatments with the other chemicals the soak method was used; that is, the cut pieces were soaked for one hour in the chemical solutions, the amounts of chemicals used being shown in the tables; the treated seed-pieces were not rinsed but were planted at once after the period of soaking.

Sampling. The time after planting at which samples were taken for the amylase measurements varied in the different tests, the intention being to let the tubers develop as far as possible and still take the sample before sprouts appeared, or at least at the time of the first evidences of sprouting.

In obtaining juice the seed-pieces were removed from the soil, were washed, wiped, peeled, and the tissue was passed through a food grinder; the minced tissue was first squeezed by hand through cheesecloth, and then subjected to further pressure by means of a small screw press. The juices from the two types of pressings were then combined and centrifuged.

Amylase measurements. The expressed juices were first brought to pH 6.2 by adding alkali or acid, using 5 cc. of acid or alkali to each 20 cc. of juice. A preliminary test indicated what strength of acid or alkali was required for this adjustment. The reaction-mixture consisted of 25 cc. of the diluted juice plus 25 cc. of 0.2 M phosphate buffer at pH 6.2, plus 50 cc. of 3 per cent soluble starch. For the check lot for each juice, 50 cc. of water instead of soluble starch were added. These mixtures were placed in large Pyrex test tubes, toluene was added, and the tubes were rotated on a turning bar inside an oven whose temperature was regulated at 35° C. After 40 hours rotation the mixtures were poured into sufficient boiling 95 per

cent alcohol to give a concentration of 70 per cent alcohol by volume. After bringing to boiling temperature the flasks were cooled, made up to volume, filtered, and an aliquot was taken; the alcohol was removed by evaporation on a water bath, and the residue was taken up with 50 cc. of H_2O; the sucrose present was inverted by the use of HCl in the cold (1, p. 95), and after neutralization and adjustment to volume, the reducing sugars present were estimated by the Munson and Walker method (1, p. 78), the cuprous oxide being titrated with potassium permanganate. The difference in the $KMnO_4$ titration values between the samples with and without starch was taken as representing the amylase activity of the juice. The values in the tables are given for aliquots representing 2.0 cc. of the original juice before the dilution for the pH adjustment.

EXPERIMENTAL RESULTS

DIRECT EFFECTS OF CHEMICALS UPON POTATO AMYLASE

By the *direct* effect of a chemical upon amylase activity is meant the influence of the chemical when it is added to the expressed juice.

TABLE I

EFFECT UPON AMYLASE ACTIVITY OF ADDING CHEMICALS TO POTATO JUICE

Ethylene chlorhydrin		Potassium nitrate		Potassium chloride	
Amount added, mg.	Amylase activity, cc. $KMnO_4$	Amount added, mg.	Amylase activity, cc. $KMnO_4$	Amount added, mg.	Amylase activity, cc. $KMnO_4$
0	10.2	0	8.8	0	8.2
250	9.1	2	8.6	2	lost
500	8.9	8	8.3	8	9.0
1000	7.3	32	8.6	32	8.8
2000	4.5	128	8.8	128	8.7
		512	8.2	512	8.9

Table I shows the effect of varying amounts of ethylene chlorhydrin, potassium nitrate, and potassium chloride upon amylase activity. Columns 1, 3, and 5 show the amounts of chemical added per 100 cc. of reaction-mixture, and columns 2, 4, and 6 the amylase values per 2.0 cc. of the original potato juice. It is seen that ethylene chlorhydrin had very little effect upon the amylase values until about 500 to 1000 mg. of anhydrous ethylene chlorhydrin had been added per 100 cc. of reaction-mixture. The range from 0 to 250 mg. had been tested previously and no effect of such amounts was observed. It is also seen from Table I that amounts of KNO_3 and KCl up to 512 mg. per 100 cc. of reaction-mixture were without effect upon the amylase values.

The direct effects of potassium cyanide, potassium thiocyanate, and sodium thiocyanate upon potato amylase were described in previous papers (2, 3).

It was shown (3) that the addition of small amounts of KCN to potato juice increased markedly the amylase activity as measured by the reducing sugars formed from the action of the juice upon soluble starch. Amounts of KCN of the order of 30 mg. per 100 cc. of reaction-mixture increased the amylase activity by 50 to 100 per cent. It was shown, however, that this increase was not observed if the juice was dialyzed in collodion bags before the addition of the KCN.

Tests of the direct effects of thiocyanates upon potato amylase as described in the previous paper (2) showed that amounts of NaSCN or KSCN in excess of about 10 mg. per 100 cc. of reaction-mixture decreased the amylase activity. In no case was an increase observed; this was true whether the juice used was that as expressed from the tuber or after dialysis in collodion bags.

Summarizing the direct effect of these chemicals upon potato amylase, we find that the cyanide hastens, the thiocyanates retard, while the nitrate, chloride, and ethylene chlorhydrin are without any pronounced effect.

INDIRECT EFFECTS OF CHEMICALS UPON POTATO AMYLASE

By the *indirect* effect of a chemical upon amylase activity is meant the influence exerted when the *tissue* is treated with the chemical and juices are obtained for the amylase test at a later period after treatment.

TABLE II

EFFECT OF CHEMICAL TREATMENT OF POTATO TUBERS UPON SPROUTING AND UPON AMYLASE (BLISS TRIUMPH VARIETY, 1930)

Chemical used	Amount of chemical per l.	Method of treatment	Amylase activity, cc. $KMnO_4$	Fresh weight of tops, g.
$ClCH_2CH_2OH$*	60.0 cc.	Dip	28.6	224
"	30.0 "	"	19.0	170
KSCN	10.0 g.	Soak		192
"	5.0 "	"	13.2	178
KCN	10.0 "	"		(Rotted)
"	5.0 "	"	11.2	74
"	2.5 "	"	21.8	74
"	1.3 "	"		36
KNO_3	50.0 "	"		96
"	40.0 "	"	12.4	58
"	30.0 "	"		36
"	20.0 "	"	14.0	32
KCl	50.0 "	"		(Rotted)
"	40.0 "	"	12.9	68
"	30.0 "	"		14
"	20.0 "	"	14.6	38
H_2O	—	Dip	13.0	3
"	—	Soak	13.0	12

* Refers to the commercial chemical which contains approximately 40 per cent ethylene chlorhydrin (by weight).

The results of such tests are shown in Tables II, III, and IV. In Table II are shown the results of a test with Bliss Triumph tubers, harvested in Maine on September 25, treated October 2, and sampled for amylase activity October 9. The amylase values, given in column 4, Table II, show that the ethylene chlorhydrin treatment induced an increased amylase activity in the expressed juice. One of the potassium cyanide treatments also showed a higher amylase value than the check, but this result was not confirmed by later tests as shown in Tables III and IV.

TABLE III

EFFECT OF CHEMICAL TREATMENT OF POTATO TUBERS UPON SPROUTING AND UPON AMYLASE (IRISH COBBLER VARIETY, 1930)

Date treated	Chemical used	Amount of chemical per l.	Method of treatment	Amylase activity, cc. $KMnO_4$	Fresh weight of tops, g.
Nov. 11	$ClCH_2CH_2OH$*	60.0 cc.	Dip		130
"	"	30.0 "	"	17.4	269
"	"	15.0 "	"		156
"	KSCN	15.0 g.	Soak		216
"	"	7.5 "	"	6.9	276
"	"	3.8 "	"		60
"	KCN	5.0 "	"		17
"	"	2.5 "	"	6.2	9
"	"	1.3 "	"		0
"	KNO_3	70.0 "	"		0
"	"	50.0 "	"	7.8	0
"	"	30.0 "	"		0
"	KCl	50.0 "	"		0
"	"	30.0 "	"	7.2	0
"	"	20.0 "	"		0
"	H_2O	—	Dip	7.9	0
"	"	—	Soak	7.5	0
Nov. 21	$ClCH_2CH_2OH$	60.0 cc.	Dip	33.7	201
"	"	30.0 "	"	32.9	136
"	KSCN	10.0 g.	Soak	11.9	384
"	"	5.0 "	"	10.4	271
"	KCN	3.3 "	"	10.1	33
"	"	1.7 "	"	8.8	71
"	KNO_3	40.0 "	"	11.8	77
"	"	20.0 "	"	10.3	34
"	KCl	40.0 "	"	11.2	100
"	"	20.0 "	"	11.2	90
"	H_2O	—	"	10.4	70
"	"	—	Dip	10.7	14
Dec. 5	NaSCN	15.0 g.	Soak	18.6	131
"	"	7.5 "	"	13.6	224
"	"	3.8 "	"	8.2	143
"	KCN	3.3 "	"		(Rotted)
"	"	1.7 "	"	7.5	74
"	"	0.9 "	"	9.4	85
"	H_2O	—		9.5	28
"	"	—		8.0	58

* Refers to the commercial chemical which contains approximately 40 per cent ethylene chlorhydrin (by weight).

In Table III are shown the results with Irish Cobbler tubers, harvested in New Jersey late in October 1930. These tubers were very dormant, sprouted slowly even after chemical treatment, and showed a slow rate of development after sprouts emerged. However, the plants were healthy. On account of their very dormant condition the tubers of this lot were favorable material for showing the comparative effects of different chemicals in influencing the rest period.

Treatments of tubers from this lot were made at three different dates, November 11, November 21, and December 5. The amylase results for the three different dates are shown in column 5, Table III. From the November 11 and November 21 treatments, samples were taken on November 19 and December 4 respectively, and it is seen that only the ethylene chlorhydrin treatment had any pronounced effect upon the amylase activity, the values for other treatments not being significantly different from those of the checks.

The test of December 5 included treatments with thiocyanate and cyanide only. In this case NaSCN instead of KSCN was used, but previous experiments on treating dormant potato tubers indicated no important differences between these two salts, the important factor being the nature of the anion and not that of the cation. The juice samples were taken on December 15, and the amylase values in column 5, Table III show that at this stage of the dormant period the sodium thiocyanate treatments caused an increase in the amylase values. The values for potassium cyanide were not greater than those of the checks, however, even at this stage. This comparison of thiocyanate and cyanide is of special interest in view of the previous results on the direct effect of these two chemicals, cyanide increasing and thiocyanate decreasing the amylase activity when the chemicals are added directly to the expressed juice. When the *tissue* is treated with the chemicals the result is quite different, cyanide not producing any increase in the amylase, and thiocyanate not producing a decrease, but, if the tubers are not too dormant, causing an increase.

TABLE IV

EFFECT OF CHEMICAL TREATMENT OF POTATO TUBERS UPON SPROUTING AND UPON AMYLASE (IRISH COBBLER VARIETY, 1931)

Chemical used	Amount of chemical per l., g.	Amylase activity, cc. $KMnO_4$			After 34 days, No. emerged out of 20 planted
		After 3 days	After 6 days	After 9 days	
NaSCN	12.50	15.6	18.0	17.8	20
"	6.25	14.7	12.3	16.6	19
"	3.13	14.7	12.3	12.5	13
KCN	2.00	18.6	11.2	12.2	11
"	1.00	14.3	10.8	13.5	5
"	0.50	13.8	11.4	14.1	5
H_2O	—	14.1	12.0	12.3	0

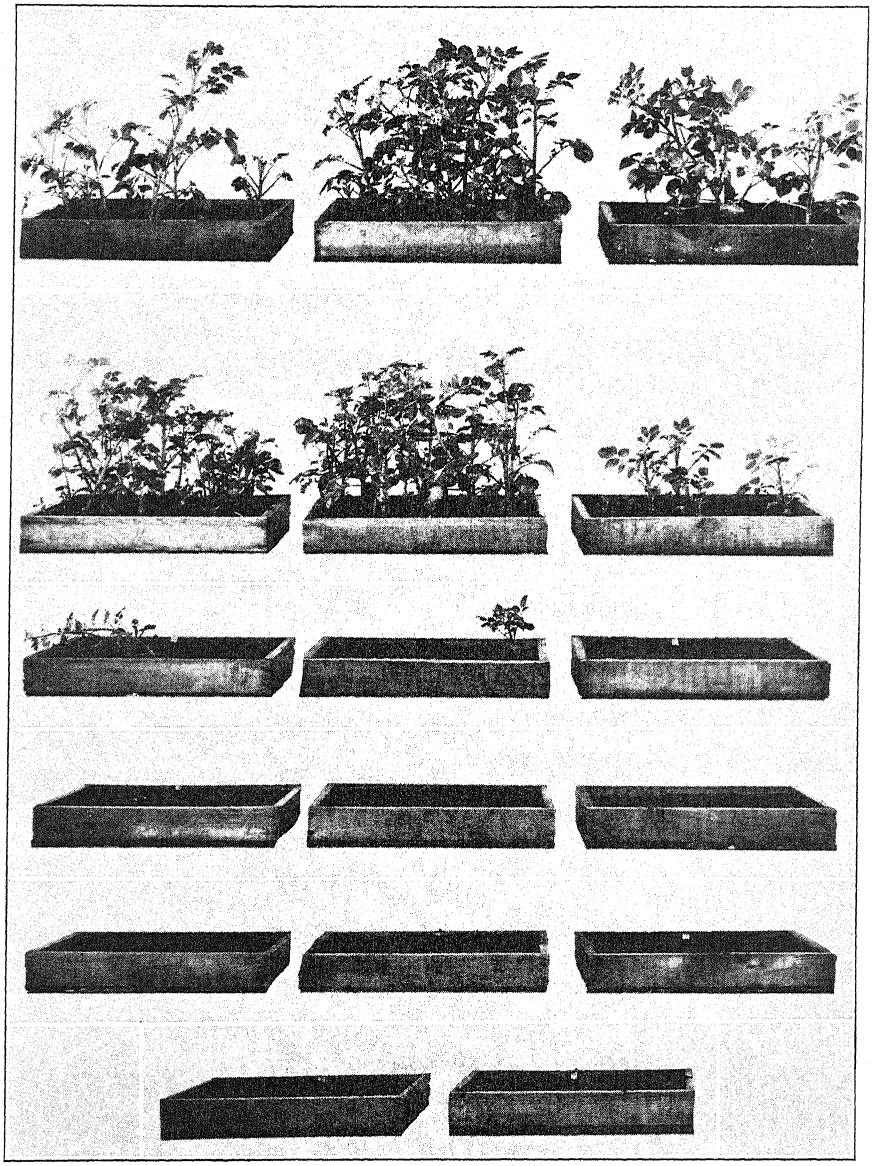

FIGURE 1. Effect of chemical treatments upon the sprouting of dormant potatoes. Subsequent growth of samples from the November 11 treatments shown in Table III. Top row, ethylene chlorhydrin treatments, left to right, the concentrations of the dipping solutions were 60 cc., 30 cc., 15 cc. per l. Second row from top, sodium thiocyanate treatments, left to right, 15.0, 7.5, 3.8 g. per l. Third row from top, potassium cyanide treatments. Fourth row, potassium nitrate treatments. Fifth row, potassium chloride treatments. Bottom pair of flats, check lots.

This point was tested in a further experiment using Irish Cobbler tubers harvested in South Carolina late in May, 1931 and treated June 16. In this experiment samples for amylase were taken on three succeeding dates: June 19, June 22, and June 25. The results are shown in Table IV. By June 25 an increase in amylase activity of at least one and perhaps two of the sodium thiocyanate treatments had occurred. But the potassium cyanide treatments did not show amylase values much greater than the check lots, and it is not certain that any increases resulted.

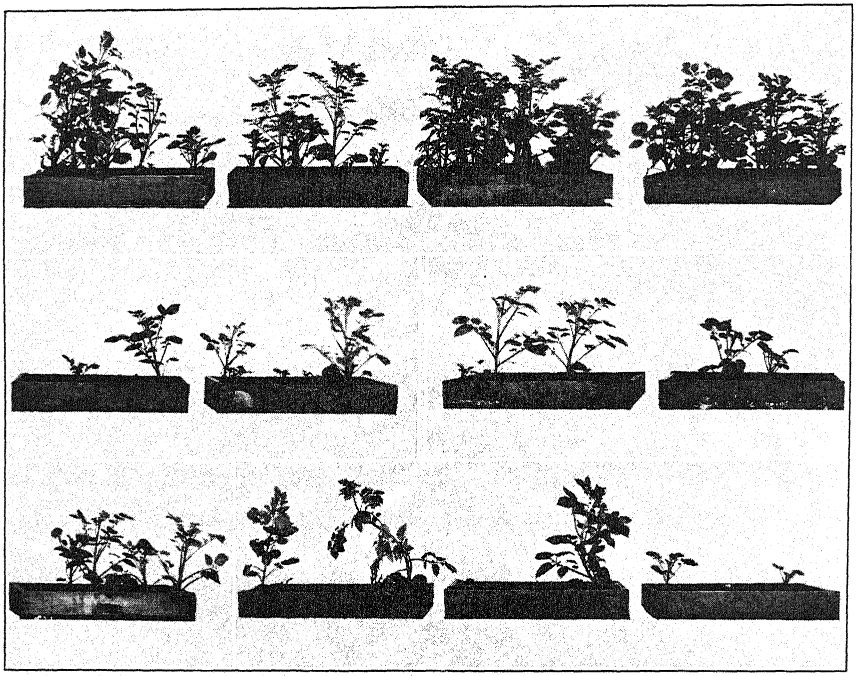

FIGURE 2. Subsequent growth of samples from the November 21 treatments shown in Table III. Top row, left to right, ethylene chlorhydrin 60 cc. and 30 cc. per l.; potassium thiocyanate 10.0 and 5.0 g. per l. Middle row, left to right, potassium cyanide 3.3 and 1.7 g. per l.; potassium nitrate 40 and 20 grams per l. Bottom row, left to right, potassium chloride 40 and 20 g. per l. and the check lots (the two flats at right in bottom row).

Summarizing the indirect effects of the chemicals upon amylase activity, it was found: that ethylene chlorhydrin treatments of the tissue induced an increase in the amylase activity; that sodium thiocyanate had much less influence upon the amylase of treated tissue, producing, however, a small increase in the activity if the treatments were applied late in the rest period; that potassium cyanide treatments of potato tissue had small and perhaps negligible effects upon the amylase activity of the ex-

pressed juice; that potassium nitrate and potassium chloride treatments were without effect upon amylase.

EFFECT OF CHEMICAL TREATMENTS UPON SPROUTING

The effect of the treatments upon the sprouting of the seed-pieces is shown in column 5, Table II, column 6, Table III, and column 5, Table IV. Also, Figures 1 and 2 show the photographs of the flats of sprouted samples from the November 11 and November 21 treatments described in Table III.

It is seen that the most favorable results were obtained with the sodium thiocyanate and ethylene chlorhydrin treatments. The other chemicals were definitely less effective in these tests. But with the Bliss Triumph variety and in the less dormant stages of the Irish Cobbler the other chemicals showed, in some cases, better germinations than the checks.

In another germination test, for which no amylase determinations were made and which are not included, therefore, in the tables, a comparison of the effectiveness of these chemicals was made. The variety was Green Mountain, harvested in Maine September 19, 1930, treated October 22, and weight of tops obtained November 24. Putting the weight of tops from the check lot at 100, the values for the most favorable treatments by the various chemicals were as follows: KSCN 725; KCN 350; KNO_3 280; KCl 250.

DISCUSSION

If we were to judge the effectiveness of a chemical for inducing sprouting by its direct effect upon amylase, we should expect cyanide to hasten, thiocyanate to retard, and ethylene chlorhydrin to have little effect. But, although all three hasten sprouting, the effect of the cyanide is distinctly less favorable than that of the thiocyanates and ethylene chlorhydrin.

If we were to judge effectiveness by the indirect effect upon amylase, we should expect only the ethylene chlorhydrin to hasten sprouting; yet cyanide and thiocyanates can hasten sprouting without having any important effect upon the amylase activity of the juice of the treated tuber.

The thiocyanates are very effective in inducing sprouting even though the indirect effect upon amylase is not particularly favorable, and in spite of its unfavorable direct effect. The cyanide hastens sprouting but not because of its favorable direct effect upon amylase since the indirect effect is not favorable to amylase increase. The extremely favorable indirect effect of ethylene chlorhydrin upon amylase is not reflected in the sprouting response, since the thiocyanates induce equally good sprouting without causing the large increase in amylase.

These considerations emphasize that the effect of the chemicals in inducing sprouting is indirect, and is not related closely to amylase ac-

tivity, nor to any effect, either direct or indirect, which the chemicals exert upon amylase activity.

SUMMARY

1. This is a report of further experiments on the effects of chemicals upon the sprouting of freshly-harvested, dormant potato tubers (*Solanum tuberosum* L.).

2. The effect upon potato amylase when chemicals were added to the expressed juice (here called the *direct* effect) was compared with the effect produced when the tissue was treated with the chemicals and the amylase activity of the juice was measured at a subsequent period (here called the *indirect* effect).

3. These effects upon amylase were then compared with the sprouting responses which were obtained when samples of the treated tubers were planted.

4. The sprouting responses were not related to any direct effect which the chemicals exerted upon amylase activity. Both sodium thiocyanate, which had a retarding action upon potato amylase if added directly to the expressed juice, and ethylene chlorhydrin which had very little effect under such conditions, hastened the germination of dormant tubers. Potassium cyanide which increased amylase activity when added to expressed juice had a much less favorable effect upon sprouting.

5. The indirect amylase effect of a chemical was not correlated with its direct effect. Thus, ethylene chlorhydrin, which had little effect upon potato amylase when it was added to the juice, induced a high amylase activity in the juice when the *tissue* was treated with the chemical and juices were expressed at a subsequent period. And potassium cyanide, whose direct effect was to increase amylase activity, did not induce the formation of a juice with high amylase activity when the *tissue* was treated.

6. The sprouting response was more nearly related to the indirect amylase effect. Thus, ethylene chlorhydrin, which induced high amylase activity in the juice if the tissue was treated, was effective in inducing sprouting; and increases in amylase activity were found to follow treatments of tissue with sodium thiocyanate, provided the potatoes were not too dormant at the time of treatment. But the correlation was not a very close one, since many of the sodium thiocyanate treatments which were effective in hastening germination did not induce any significant increase in the amylase activity of the tissue.

7. Tests were also made with potassium nitrate and potassium chloride, but these had no important effects upon amylase in either a direct or indirect manner. Their effects upon sprouting, also, were not striking.

8. The results of these experiments corroborate the view that the effects of the chemicals in inducing sprouting are *indirect*, and are not due to *direct* effect upon the amylase activity; they indicate further that

capacity to sprout is not dependent upon the development of high amylase activity of the juice of the treated tissue.

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CHANGES IN LEAVES DURING THE NIGHT

F. E. DENNY

INTRODUCTION

In attempting to measure the changes which occur in leaves during the night it is recognized that the basis of calculation has an important bearing upon the reliability of the data. If the amount of the constituent is expressed as a percentage of the fresh weight or dry weight, the calculated change is a true one only if the fresh weight or dry weight has not changed during the period. In leaves it is rarely found that either the fresh weight or dry weight is constant during the night, the tendency being for the fresh weight to increase and for the dry weight to decrease.

In like manner when the amount of the constitutent is expressed on the basis of the area of the leaf the calculated result is uncertain until it is known whether the leaf has undergone shrinkage or distention because of change in the water content. Usually the leaf gains in water during the night, and certainty regarding a change in any constituent cannot be reached because of our lack of knowledge as to the relation between changes in water content of a leaf and changes in its area.

In the present experiments an effort has been made to measure the changes in leaves by the use of methods in which the disturbance caused by changes in water content can be eliminated as a factor in making computations.

In a previous paper (5) results with the use of the twin-leaf method for measuring changes in leaves were given. By this method pairs of leaves of plants having opposite leaves or leaflets are selected, one leaf of each pair being removed at the start of the experiment and the other removed at the end. The success of this method depends upon the uniformity of opposite leaves, but experience shows that the error in assuming that the leaves were equal at the start can be kept to a low value by the selection of suitable material and by including the proper number of pairs in the test. The advantage of this method is that percentages of constituents need not be dealt with and the data can be given in absolute values. Changes in area, water content, fresh weight or dry weight need then have no influence upon the calculations; indeed these factors themselves can be measured and expressed in absolute values by this method.

In a similar way the half-leaf method can be used to obtain absolute values. By this method one-half of a leaf is removed by cutting along the midrib and the other half is allowed to remain until the end of the experiment. This method has been criticized because of the possible effects of mutilation, and, while this may be an important factor in certain cases, in the present experiment the results do not justify any serious questions as

to dependability. It is believed, however, that the half-leaf method has been used improperly in certain previous experiments, in that the authors have not dealt with the absolute values of the half leaves but have expressed their data on the area basis, thereby introducing into their calculations the error it is necessary to avoid, i.e., the possible change in leaf area during the experiment.

Finally we can use whole leaves instead of twin leaves or half leaves and still obtain not absolute values but at least relative values by expressing the data upon some fraction of the tissue that does not change in weight during the course of the experiment. Mason and Maskell (7) suggested the use of the residual dry weight (dry weight minus total carbohydrate) as a basis for calculation. This method was tested in the present experiments and found satisfactory. In addition, measurements are presented showing to what extent the residual dry weight may be regarded as a constant in the samples under comparison, and, therefore, to what extent the calculated results are dependable.

These experiments were undertaken not merely to study the use of these three methods in determining diurnal changes in leaves but also to measure some of the changes that were taking place. The literature of the subject shows fairly good agreement in favor of the view that during the night there is a loss of dry weight and carbohydrate material from the leaves, and, furthermore, that the nitrogen metabolism resembles that of carbohydrate, i.e., that protein is deposited in the leaves during the day, is broken down during the night, and is translocated from the leaf to other parts of the plant.

The present results suggest modification of these views in the following respects: (a) Loss of dry weight from leaves during the night is pronounced in some species, but in other species the loss may be so small as hardly to be detectable; woody in contrast to herbaceous plants show these low losses during the night. (b) Losses of nitrogen from leaves during the night have been low in the present experiments in those cases in which any loss at all was found; and in many cases no losses in nitrogen were found; it is believed that the theory of a diurnal change in nitrogen analogous to that of carbohydrate has not been supported at the present time with sufficient evidence to establish it conclusively.

METHODS

Species used. The species used were as follows: tobacco, *Nicotiana tabacum* L.; sunflower, *Helianthus annuus* L.; cotton, one experiment with the strain Super Seven of the species *Gossypium hirsutum* L., and another experiment in which plants of various strains and species were included; hawthorn, *Crataegus mollis* (T. and G.) Scheele; Virginia creeper, *Psedera quinquefolia* (L.) Greene; redbud, *Cercis canadensis* L.; lilac, *Syringa vul-*

garis L. variety Charles X; Scarlet Runner bean, *Phaseolus coccineus* L.; lima bean, *Phaseolus limensis* Macf. var. Fordhook; peach, *Prunus persica* (L.) Stokes; garden bean, *Phaseolus vulgaris* L., varieties Marrow, Cutshort, and Henderson's Stringless; soybean, *Glycine max* Merr., varieties Biloxi and Tokio; *Salvia splendens* Ker.; grape, *Vitis labruscana* Bailey, var. Concord.

Collection of samples. Only leaves that appeared to have reached a mature condition were selected. They were weighed at once after removal from the plant to obtain the fresh weight. If there had been a rain or heavy dew during the night the leaves were wiped first with blotting paper and with dry cheesecloth. Footnotes are given in the various tables showing in which cases it was necessary to obtain fresh weights after rain or dew, thus allowing the reader to judge whether the fresh weight values were seriously in error because of this unavoidable difficulty. After the fresh weight was recorded the leaves were dropped into vigorously boiling ethyl alcohol in order to inactivate enzymes. The entire sample was then transferred to tared glass beakers, the alcohol was evaporated on a water-bath, and the dry weight was obtained on the residue after it had been in an electric oven at 96° C. for 16 hours. The dry tissue was then powdered and samples were taken for analysis.

Analytical methods. Each sample was extracted with hot 70 per cent ethyl alcohol (by volume) in a Pyrex centrifuge tube, was centrifuged after each extraction, and was subjected to seven successive extractions. The alcoholic solutions from the various extractions of each tissue were combined and aliquots were taken for the soluble nitrogen and for total sugar. For the soluble nitrogen the method of Pucher, Leavenworth, and Vickery (8), which includes nitrate nitrogen, was used. For the sugar determinations neutral lead acetate was added to the aqueous solution obtained after evaporation of the alcohol and the excess lead was removed with potassium oxalate. Total sugar was determined on this liquid after inversion with hydrochloric acid in the cold (1, p. 95). For the sugar determinations the Munson and Walker method was used (1, p. 78) and the cuprous oxide was titrated with a potassium permanganate solution which had been standardized with a sugar solution of known concentration. The residues after extraction with alcohol were used for the insoluble nitrogen, polysaccharide, and starch determinations. Insoluble nitrogen was determined by the Gunning method (1, p. 7). The polysaccharides were estimated by the acid hydrolysis method (1, p. 95) and represent the alcohol-insoluble, acid-hydrolyzable substances calculated as dextrose. The polysaccharide values included starch but a separate determination of starch was made by the Walton and Coe (10) method which eliminates interfering polysaccharides. This method was modified by using saliva instead of malt diastase. The starch values are probably low since it was found that the

TABLE I

CHANGES IN LEAVES DURING THE NIGHT. GREENHOUSE EXPERIMENTS USING THE TWIN-LEAF METHOD

A. DIURNAL SAMPLES

Name of plant	Time of sampling	No. of leaves	Total amount in sample: Fresh wt. g.	Dry wt. g.	Sol. N. mg.	Insol. N. mg.	Total N. mg.	Per cent gain (+) or loss (−) during the period: Fresh wt.	Dry wt.	Sol. N.	Insol. N.	Total N.
Salvia	p.m.	50	30.89	4.600	14.8	172.0	186.8	+4.5	−6.5	+1.3	+0.9	+1.3
	a.m.	50	32.28	4.302	15.0	173.6	188.6					
	p.m.	50	31.15	4.721	26.3	163.2	189.5	+4.2	−8.2	−8.4	+3.9	+2.2
	a.m.	50	32.46	4.336	24.1	169.6	193.7					
	p.m.	50	30.07	4.860	15.6	150.8	166.4	+13.0	−30.1	+4.5	−3.5	−2.7
	a.m.*	50	34.00	3.395	16.3	145.6	161.9					
	p.m.	50	30.47	4.211	15.1	157.4	172.5	+6.5	−22.2	+5.8	−8.8	−3.0
	a.m.**	50	32.45	3.273	23.8	143.6	167.4					
	p.m.†	50	31.50	4.955	8.9	126.6	135.5	+1.3	−14.7	+33.8	−2.7	−0.3
	a.m.†	50	31.92	4.223	11.9	123.2	135.1					
	p.m.	50	34.69	5.167	10.1	162.8	172.9	+2.9	−26.4	+123.8	−8.2	−0.5
	a.m.††	50	35.71	3.804	22.6	149.4	172.0					
Soybeans	p.m.	62	8.69	1.669	19.1	86.0	105.1	+25.8	−12.6	+6.3	+0.7	+1.7
	a.m.	62	10.95	1.457	20.3	86.6	106.9					
	p.m.	95	11.51	2.343	17.0	121.0	138.0	+9.9	−13.2	+2.4	−2.5	−1.9
	a.m.	95	12.65	2.033	17.4	118.0	135.4					
	p.m.	68	11.26	2.460	8.3	89.6	97.9	+5.1	−14.6	−12.1	−2.6	−3.4
	a.m.	68	11.83	2.100	7.3	87.3	94.6					
	p.m.	99	13.71	3.068	8.5	129.0	137.5	+2.8	−17.8	−15.3	−2.3	−3.1
	a.m.	99	14.10	2.519	7.2	126.0	133.2					
Cutshort beans	p.m.	88	15.26	2.494	6.4	91.5	97.9	+4.4	−9.3	−17.2	+0.8	−0.4
	a.m.	88	15.93	2.262	5.3	92.2	97.5					
	p.m.	75	10.62	1.558	7.1	64.6	71.7	+6.1	−6.4	−9.9	0	−1.0
	a.m.	75	11.27	1.458	6.4	64.6	71.0					
	p.m.	62	11.01	1.814	21.6	90.8	112.4	+30.5	−12.8	−4.2	+2.0	+0.8
	a.m.	62	14.39	1.580	20.7	92.6	113.3					
	p.m.	63	15.89	2.471	11.7	95.8	107.5	+2.6	−16.6	−17.9	−5.9	−7.2
	a.m.	63	16.31	2.060	9.6	90.2	99.8					

* After 66.5 hrs. in the dark; ** after 5 days in the dark.

† Older plants distinctly pot-bound; †† pot-bound plants held 5 days in the dark.

TABLE I (*continued*)
B. SIMULTANEOUS SAMPLES

Name of plant	Time of sampling	No. of leaves	Total amount in sample: Fresh wt. g.	Dry wt. g.	Sol. N. mg.	Insol. N. mg.	Total N. mg.	Per cent difference between simultaneous samples: Fresh wt.	Dry wt.	Sol. N.	Insol. N	Total N.
Salvia	Simultaneous twin leaf samples taken either both in the p.m. or both in the a.m.	50 50	27.43 27.25	3.253 3.212	14.0 13.4	146.8 150.6	160.8 164.0	0.7	1.3	4.3	2.6	2.0
		50 50	28.69 29.01	3.474 3.503	14.0 13.4	156.0 162.2	170.0 175.6	1.1	0.8	4.3	4.0	3.3
		50 50	25.45 25.47	3.058 3.084	13.4 12.7	136.0 136.2	149.4 148.9	0.1	0.9	5.2	0.1	0.3
		50 50	30.73 30.79	3.123 3.136	20.2 21.7	162.4 157.0	182.6 178.7	0.2	0.4	7.4	3.3	2.1
		50 50	28.51 28.45	3.081 3.081	16.2 17.3	137.0 147.6	153.2 164.9	0.2	0	6.8	7.7	7.6
		50 50	30.78 31.41	3.362 3.393	21.3 21.6	169.8 169.4	191.1 191.0	2.0	0.9	1.4	0.2	0
Soybeans		34 34	6.05 6.15	1.065 1.075	11.4 11.5	56.2 54.8	67.6 66.3	1.7	0.9	0.9	2.5	1.9
		45 45	8.53 8.43	1.535 1.528	14.9 13.4	76.4 78.2	91.3 91.6	1.2	0.5	10.0	2.4	0.3
		74 74	11.28 11.54	2.571 2.639	16.5 18.0	106.4 109.4	122.9 127.4	2.3	2.6	9.1	2.8	3.7
		55 55	8.45 8.31	1.948 1.910	13.5 12.8	72.4 73.6	85.9 86.4	1.7	1.9	5.2	1.7	0.6
Cutshort beans		64 64	13.97 13.96	2.525 2.523	16.4 17.4	103.8 102.4	120.2 119.8	0.1	0.1	6.1	1.3	0.3
		61 61	12.60 12.15	2.164 2.075	12.2 12.2	96.4 93.0	108.6 105.2	3.6	4.1	0	3.5	3.1
		63 63	12.62 12.78	2.105 2.118	8.3 8.8	95.0 92.0	103.3 100.8	1.3	0.6	6.0	3.2	2.4

residue even after 48 hours digestion with enzyme often gave tests for undigested starch.

RESULTS

WITH THE TWIN-LEAF METHOD

Greenhouse experiments. These were carried out during the period from May 1 to June 18, 1931. The plants were grown in pots and the leaves selected for experiment were such as had attained approximately full size. In selecting the leaves alternately right and left leaves of each pair were taken for the afternoon sample. After the twin leaves representing the p.m. sample had been collected, usually at about 3 to 4 o'clock p.m., the potted plants were placed in a dark space obtained by covering a wooden frame with black cloth. The plants were allowed to remain in this space until about 8:15 a.m. the next day, at which time the twin leaves corresponding to those taken for the p.m. samples were collected for analysis. Salvia, soybeans, and Cutshort beans were used in these tests. The results are shown in Table I. The values given in columns 4 to 8 show the total weights for the entire sample in each case, and columns 9 to 13 show the per cent gain or loss during the night. Table I (continued) gives the results with simultaneous samples of the same species of plants; it shows the values that are obtained when samples of twin leaves are taken simultaneously but subjected otherwise to the same procedure as those taken in the evening and in the morning. The per cent differences in the right hand columns of the table show the sampling errors under these conditions; these differences by simultaneous sampling can be compared with the differences shown by diurnal sampling.

The gains in fresh weight and losses in dry weight during the night were clearly too large in most cases to be accounted for by the sampling error. The nitrogen data, however, were not conclusive in showing either gains or losses during the night. The total nitrogen values are within the error of sampling. In a test with salvia (see lines 7 and 8 in Table I) in which the plants were allowed to stay five days in the dark room there was probably a loss in insoluble nitrogen. And, when plants were allowed to grow for a longer time in pots until they became pot-bound and were showing signs of distress, then as shown in lines 9, 10, 11, and 12, Table I, there was evidence that the insoluble nitrogen decreased and that the soluble forms increased, particularly after five days in the dark. But even in these cases the total nitrogen did not decrease; there was a breakdown of the insoluble nitrogen to soluble forms but these were not transported from the leaf.

Outdoor experiments. These results are shown in Table II. The Marrow bean, Stringless bean, and lima bean samples were taken July 13–14 from plants growing in the garden; the p.m. samples were collected at 6:00

TABLE II

CHANGES IN LEAVES DURING THE NIGHT. OUTDOOR EXPERIMENTS USING THE TWIN-LEAF METHOD

A. DIURNAL SAMPLES

Name of plant	Time of sampling	No. of leaves	Total amount in sample: Fresh wt. g.	Dry wt. g.	Sol. N. mg.	Insol. N. mg.	Poly-sacch. g.	Starch g.	Total sugar g.	Per cent gain (+) or loss (−) during the period: Fresh wt.	Dry wt.	Total N.	Total-carbo-hydrates
Scarlet runner bean	p.m.	100	89.00	17.61	62.2	634	2.393	1.708	0.757	+6.1	−8.5	−1.7	−36.1
	a.m.	100	94.40	16.11	53.5	631	1.564	0.768	0.448				
Marrow bean	p.m.	40	30.65	6.05	19.1	214	1.168	0.967	0.233	+3.8	−13.5	+2.1	−46.0
	a.m.	40	31.82	5.23	18.1	220	0.584	0.355	0.171				
Stringless bean	p.m.	36	38.80	6.64	21.2	242	1.052	0.746	0.226	+7.3	−10.2	−2.7	−54.8
	a.m.*	36	41.64	5.96	20.0	236	0.444	0.219	0.132				
Lima bean	p.m.	103	104.0	17.55	63.1	604	2.955	2.110	0.537	+5.0	−12.5	+4.1	−56.2
	a.m.	103	109.2	15.36	63.6	631	1.121	0.663	0.416				
Lilac	p.m.	50	56.88	18.27	38.6	465	1.604	0.636	1.573	+5.5	−0.3	−1.4	−4.5
	a.m.	50	60.03	18.22	33.8	463	1.519	0.440	1.516				

B. SIMULTANEOUS SAMPLES

(Per cent difference between simultaneous samples)

Name of plant	Time of sampling	No. of leaves	Fresh wt. g.	Dry wt. g.	Sol. N. mg.	Insol. N. mg.	Poly-sacch. g.	Starch g.	Total sugar g.	Fresh wt.	Dry wt.	Total N.	Total-carbo-hydrates
Scarlet runner bean	Simultaneous samples of half leaves taken either both in a.m. or both in p.m.	100	87.76	16.02	57.8	615	2.137	1.501	0.646	1.6	0	1.4	3.4
		100	86.38	16.02	57.3	606	2.248	1.539	0.631				
Marrow bean		36	28.68	5.44	16.7	203	0.904	0.836	0.253	2.5	1.3	0.2	10.0
		36	27.96	5.37	17.2	202	1.008	0.809	0.265				
Stringless bean		36	42.59	7.13	21.4	263	0.946	0.696	0.299	2.1	2.2	1.5	2.3
		36	41.68	6.97	21.1	259	0.990	0.646	0.284				
Lima bean		65	72.45	12.27	45.4	447	1.895	1.224	0.431	1.0	1.0	0.4	6.1
		65	71.72	12.15	45.0	445	2.070	1.326	0.398				
Lilac		50	53.45	18.38	29.5	468	1.690	0.687	1.533	0.2	0.3	1.5	1.1
		50	53.57	18.44	32.2	473	1.679	0.698	1.510				

* Rain during night.

to 7:00 p.m., and the a.m. samples at 6:00 to 6:30 a.m. These plants were full grown and were forming pods at the time. The Scarlet Runner beans were full grown plants about five feet high in bloom and entwined around poles; samples were taken July 27. The lilac samples were taken August 31 from plants about four feet high. The data in columns 4 to 10 show the total amounts of substances present in the entire sample, and columns 11 to 14 show the per cent gain or loss of certain constituents during the night. The data for the simultaneous samples are shown at the bottom of Table II and represent the sampling errors obtained under such conditions. The results with beans show clearly that the fresh weights had gained and the dry weights had lost during the night. The large losses in polysaccharides, starch, and total sugar were much in excess of the differences in the simultaneous samples. Approximately one-half of the total carbohydrate disappeared from the leaves during the night. The changes in nitrogen, however, were small and in view of the differences shown by the simultaneous samples were not sufficient to show that any loss in total nitrogen from the leaves had occurred. The data for the lilac leaves are interesting in showing that, although there may have been a gain in fresh weight and a small loss in starch, the changes in the other constituents were so small as to require further evidence that any real change occurred.

WITH THE HALF-LEAF METHOD

The experiments with Virginia creeper, sunflower, and redbud were carried out July 22–23, and those with cotton and grape August 31–September 1. The sunflower and cotton plants were grown in pots and the experiment was carried out in the greenhouse; the samples from other species were taken from plants growing in the garden and grounds of the Institute. The p.m. samples were taken late in the day and the a.m. samples early the next morning. In cutting off the half leaves for the evening sample right and left halves were taken alternately. The half leaves were removed by cutting with a razor close to the midrib which was finally discarded. The results are shown in Table III; the amounts in columns 4 to 10 give the total weights of the entire sample and the values in columns 11 to 14 represent the per cent gain or loss during the night. It is seen that the sunflower showed large changes in all constituents except nitrogen; for Virginia creeper, grape, and redbud, however, the principal percentage losses occurred in the carbohydrate fractions; the dry weight losses were not large and the fresh weight underwent no indisputable change. The nitrogen changes were in all cases within the error of sampling.

In this test we note that the woody types show low changes in leaves during the night. Whether this is a general distinction between herbaceous and woody plants, and whether this difference is due to lower translocation or lower respiration or both must be left for future experiments.

TABLE III

CHANGES IN LEAVES DURING THE NIGHT. EXPERIMENTS USING THE HALF-LEAF METHOD

A. DIURNAL SAMPLES

Name of plant	Time of sampling	No. of half leaves	Total amount in sample: Fresh wt. g.	Dry wt. g.	Sol. N. mg.	Insol. N. mg.	Poly-sacch. g.	Starch g.	Total sugar g.	Per cent gain (+) or loss (−) during the period: Fresh wt.	Dry wt.	Total N.	Total carbo-hydrates
Virginia creeper	p.m. a.m.*	36 36	18.11 18.10	5.43 5.30	7.0 7.1	118 118	0.651 0.499	0.391 0.233	0.123 0.101	0	−2.4	+0.1	−22.5
Sunflower	p.m. a.m.	42 42	42.42 48.54	6.20 5.54	15.5 15.1	283 277	0.847 0.355	0.435 0.088	0.120 0.033	+14.4	−10.6	−2.1	−59.8
Grape	p.m. a.m.**	36 36	77.13 83.56	26.70 25.90	19.8 20.1	650 646	2.840 2.440	0.897 0.609	0.754 0.664	+8.3	−3.0	−0.5	−13.6
Cotton	p.m. a.m.	36 36	54.53 54.27	11.51 10.96	43.6 46.9	420 418	1.355 1.041	0.777 0.414	0.244 0.169	−0.5	−4.8	+0.3	−24.3
Redbud	p.m. a.m.*	30 30	19.31 19.52	6.78 6.44	14.3 11.8	206 209	0.679 0.506	0.434 0.137	0.103 0.060	+1.1	−5.0	+0.2	−27.6

B. SIMULTANEOUS SAMPLES

Name of plant	Time of sampling	No. of half leaves	Fresh wt. g.	Dry wt. g.	Sol. N. mg.	Insol. N. mg.	Poly-sacch. g.	Starch g.	Total sugar g.	(Per cent difference between simultaneous samples): Fresh wt.	Dry wt.	Total N.	Total carbo-hydrates
Virginia creeper	Simultaneous samples of half-leaves taken either both in a.m. or both in p.m.	36 36	15.47 15.61	4.97 5.01	5.4 5.5	109 109	0.513 0.539	0.289 0.292	0.062 0.054	0.9	0.8	0.1	3.1
Sunflower		42 42	37.84 39.08	5.50 5.66	12.8 13.2	260 272	0.720 0.725	0.319 0.344	0.109 0.105	3.3	2.9	4.5	0.1
Grape		36 36	75.58 74.03	28.16 27.29	20.3 19.7	684 656	2.482 2.605	0.777 0.729	0.743 0.677	2.0	3.1	4.1	1.8
Cotton		36 36	49.05 48.63	10.65 10.57	45.1 48.9	377 386	1.082 1.042	0.455 0.463	0.252 0.275	0.9	0.7	3.0	1.3
Redbud		30 30	16.11 16.33	5.46 5.51	11.3 11.9	178 177	0.616 0.667	0.088 0.069	0.069 0.061	1.4	0.9	0.2	6.3

* Rain during night; ** heavy dew in a.m.

Lilac leaf experiment. The data in Table II for lilac and in Table III for Virginia creeper, grape, and redbud showing small changes during the night with these types are substantiated by additional tests with lilac leaves. Five further tests with lilac leaves were made between June 22 and July 28, 1931. The results are shown in Table IV. There may have been an increase in fresh weight but it is seen that the dry weight and nitrogenous fractions show practically no change during the night.

TABLE IV

CHANGES IN LILAC LEAVES DURING THE NIGHT
HALF-LEAF METHOD
A. DIURNAL SAMPLES

Time of sampling	No. of half leaves	Total amount in sample				Per cent gain (+) or loss (−) during the period		
		Fresh wt. g.	Dry wt. g.	Sol. N. mg.	Insol. N. mg.	Fresh wt.	Dry wt.	Total N
p.m. a.m.	32 32	8.92 9.66	2.86 2.81	4.5 5.5	75.0 73.0	+8.3	−1.7	−1.3
p.m. a.m.*	36 36	13.07 13.64	3.85 3.86	5.8 6.3	90.0 90.5	+4.4	+0.3	+1.0
p.m. a.m.*	36 36	15.46 15.14	4.48 4.45	6.9 6.2	105.9 105.0	−2.1	−0.7	−1.4
p.m. a.m.	36 36	11.96 12.76	3.70 3.71	5.2 6.1	87.0 87.6	+6.8	+0.3	+1.6
p.m. a.m.	36 36	14.13 14.79	4.21 4.17	5.8 5.8	99.7 96.9	+4.7	−1.0	−2.7
		B. SIMULTANEOUS SAMPLES				Per cent difference between simultaneous samples		
Simultaneous samples of half leaves taken either both in a.m. or both in p.m.	28 28	6.85 6.96	2.03 2.04	5.5 4.5	56.3 57.3	1.6	0.5	0
	36 36	9.75 9.66	3.17 3.17	3.9 4.3	74.0 74.8	0.9	0	1.5
	36 36	12.73 12.86	3.79 3.83	5.7 5.4	89.2 90.8	1.0	1.1	1.4
	36 36	11.94 12.21	3.63 3.54	6.0 5.0	84.4 82.4	2.3	2.5	3.3

* Rain during the night.

WITH WHOLE LEAVES BY THE RESIDUAL-DRY-WEIGHT-METHOD

The two methods previously described, the twin-leaf and half-leaf methods, can be used only with certain kinds of leaves, those with opposite leaves sufficiently uniform in size to permit the collection of good samples, or those with opposite halves showing symmetry of size and shape. It

would be desirable to have a dependable method in which these requirements need not be met so that leaves of various shapes and sizes could be used. Tests were therefore made of the residual-dry-weight-method first proposed by Mason and Maskell (7). By this method the basis of computation is the value obtained by subtracting the weight of total carbohydrates from the dry weight.

The thesis is that, if it is mainly the carbohydrates that are undergoing change in amount during the period of observation, the residual matter after subtracting the carbohydrates represents a relatively stable and constant fraction; calculations on the basis of this unchanging residue should give a true measure of the relative change in two different samples.

Mason and Maskell presented no data to indicate how nearly constant this residual matter may be over a period of time. The data in Tables II and III in the present experiments provide us with measurements by which we can test whether the assumption that the residual dry weight has remained constant from night to morning is true. Since the twin leaves and half leaves selected for comparison in each experiment were approximately equal at the time the night sample was taken, if the sum of the polysaccharides and sugars is subtracted from the total dry weight, we should obtain the same weight for the night and morning samples.

This calculation has been made for the ten different species of plants dealt with in Tables II and III and is presented in Table V. The figures in column 3 in Table V show the weights obtained by subtracting the sum of the polysaccharides and total sugars from the dry weights in the cases of the p.m. samples; and column 4 shows the corresponding calculation for the a.m. samples. Column 5 shows the per cent difference between these two sets of values, i.e., shows the error involved in assuming that the residual dry weight has remained constant during the night. The average error is only about two per cent.

TABLE V

RESIDUAL DRY WEIGHTS AT NIGHT AND IN MORNING

Name of plant	Diurnal samples				Simultaneous samples			
	No. of leaves	Residual dry wt. p.m.	Residual dry wt. a.m.	Per cent diff.	No. of leaves	Residual dry wt. a	Residual dry wt. b	Per cent diff.
Scarlet runner bean	100	14.46	14.10	2.5	100	13.24	13.14	0.8
Marrow bean	40	4.65	4.47	3.9	36	4.28	4.10	4.2
Stringless bean	36	5.36	5.38	0.4	36	5.88	5.70	3.1
Lima bean	103	14.06	13.82	1.7	65	9.94	9.68	2.6
Lilac	50	15.09	15.18	0.6	50	15.16	15.25	0.6
Virginia creeper	36	4.66	4.70	0.9	36	4.39	4.42	0.7
Sunflower	42	5.23	5.15	1.5	42	4.67	4.83	3.4
Grape	36	23.11	22.80	1.3	36	24.92	24.01	3.6
Cotton	36	9.91	9.75	1.6	36	9.32	9.25	0.8
Redbud	30	6.00	5.87	2.2	30	4.77	4.78	0.2

Furthermore, in the right hand half of Table V are shown the corresponding data for the simultaneous samples; the last column shows that the error of simultaneous samples is itself about two per cent, approximately equal to the error of the diurnal samples. This indicates that the residual dry weights of these samples have not changed essentially during the period of observation, and that the percentage of constituents calculated on this value should give a good basis for comparison.

By means of the twin-leaf and half-leaf methods, therefore, we are able to obtain evidence that Mason and Maskell's proposal was founded upon a correct assumption, and that calculations made upon this basis may be used with confidence. Since it has been found to be valid for these ten types of plants in which twin leaves and half leaves may be obtained, we may believe that it would be applicable to types of leaves not suitable for sampling by these two methods, and probably to leaves in general.

TABLE VI

ILLUSTRATING METHOD OF COMPUTING BY RESIDUAL-DRY-WEIGHT-METHOD

Constituent	Amount per gram dry wt. g.	Amount per gram residual-dry-wt. g.
Fresh wt.	6.39	7.64
Dry wt.	1.000	1.196
Sol. N.	0.00435	0.00520
Insol. N.	0.0406	0.0485
Polysacch.	0.140	0.167
Starch*	0.111	0.133
Total sugar	0.0236	0.0282
Residual dry wt.	0.8364	1.000

* Starch included in the polysaccharide determination.

Tests with the residual-dry-weight-method using whole leaves (including the midribs) were carried out between July 23 and August 31. The results are shown in Tables VI and VII. It will be noted that the data in columns 3 to 9 in Table VII are expressed in amounts per gram of residual dry weight; the procedure by which these values were obtained will be described by taking as an illustrative case the tobacco sample in the top line. The analytical results are listed first as grams of each constituent per gram of dry leaf powder as shown in column 2, Table VI. The residual dry weight (0.8364) is obtained by subtracting the sum of 0.140 and 0.0236 from 1.000. Each value in column 2, Table VI is then divided by 0.8364, which gives the values in column 3, Table VI. It is in this way that the data in columns 3 to 9, Table VII were obtained from the analyses of the samples of powdered leaves.

It will be noted in Table VII that each p.m. and each a.m. is represented by two separate samples. The average of each pair is taken as the repre-

sentative value for each p.m. and a.m. period. The difference between these two is expressed as a per cent of the p.m. value; these values are listed in columns 10, 12, 14, and 16 in Table VII and represent the per cent gains or losses during the night. Columns 11, 13, 15, and 17 show the average per cent total differences between the pairs of simultaneous samples. This permits an estimate to be made of the sampling error, and while there are too few simultaneous samples of any one species of plant to provide a very accurate value for the error, it at least gives a rough measure, and permits a comparison of the diurnal differences with the differences obtainable by simultaneous sampling.

The evidence is in favor of the view that the fresh weight increased during the night, although the data with grape and peach were rendered less conclusive because of interference by rain. The dry weight values show losses during the night for all tests with the possible exception of those with peach and hawthorn. Even with them, however, probably small losses occurred. The total carbohydrate losses expressed as percentages of the amount present in the p.m. sample were high in all cases except those of peach and hawthorn. The nitrogen changes were small and hardly conclusive except possibly with the test with the cotton plants that were allowed to retain their bolls. In general, however, evidence in favor of a diurnal change in nitrogen is not conclusive.

From the results in Table VII we are able again to distinguish between herbaceous types such as sunflower and tobacco and woody types such as peach and hawthorn. It will be noted that relatively large changes occurred in the sunflower and tobacco and small ones in the peach and hawthorn. Grape and cotton were intermediate in behavior, grape tending to resemble the woody type and cotton the herbaceous type.

DISCUSSION

Fresh weight. In nearly every experiment the fresh weight increased during the night in spite of the fact that the dry matter usually decreased. This increase in fresh weight would make calculations upon that basis entirely misleading; for even though a constituent should undergo no change at all during the night, the calculation upon the fresh weight would show a loss. Practical considerations also make it inadvisable to use fresh weight as a basis, since rain or a heavy dew between sampling periods makes the determination of the fresh weight subject to doubt.

Dry weight. It was shown that the dry weight losses were very high with some species and so low with others as to be hardly measurable. Whether these losses were due mainly to a translocation of substances from the leaf to other parts of the plant or resulted from leaf respiration is not dealt with in this paper. In some papers it has been assumed apparently that the loss results mainly from translocation, but in at least one paper,

that of Tollenaar (9), it is suggested that the loss is mainly due to respiration, that at least four-fifths of the substances that disappear do not pass out of the leaf but are used up in its own respiration. From a recent paper by Crafts (4) we find measurements regarding the relative loss by translocation and respiration; these indicate that the loss by respiration is lower than Tollenaar's estimate. Further experiments upon this phase of the question are needed.

Nitrogen. Most of the previous measurements of nitrogen changes in leaves during the night have indicated a definite loss in total nitrogen. As for those experiments in which the calculations have been made upon the fresh weight or upon the leaf area basis we are justified in withholding full agreement because of doubt as to the constancy of the fresh weight or leaf area during the period of observation. Against the experiments of Chibnall (3) and of Maskell and Mason (6), however, these objections cannot be made. Maskell and Mason's computations were made upon the residual-dry-weight-method, and, although Chibnall preferred the fresh weight basis for his estimates he used twin leaves and records the absolute values. Hence Chibnall's values, in so far as they relate to the total weight per hundred leaves, should be comparable with the results in the present experiments. Chibnall's Table VIII, p. 393, shows a loss of 4.1 per cent of the total nitrogen during the night. The least satisfactory part of Chibnall's experiments is that he found losses in nitrogen but no changes in dry weight. This is not a normal condition; changes in nitrogen should have been accompanied by much larger changes in dry weight. As he himself says in a previous article (2, p. 513) "But of the substances leaving the leaf through the petiole only part, probably only a small part, will be nitrogen." Chibnall recognized this defect in his data and suggested that the gain in weight during the early morning period of light (about one and one-half hours) might have made up for the losses at night. This would require that the rate of synthesis during this short period be much faster than the breakdown during the night; would require that very little nitrogen be brought into the leaf during this time; and that the gain in dry weight in the early morning period be such as to balance precisely the losses in nitrogenous compounds during the night. It is believed that Chibnall's experiments represented unusual conditions, and that a translocation of nitrogen from leaves in general during the night cannot be established conclusively by them. Maskell and Mason (6) present curves showing the nitrogen changes in leaves using calculations based on both the fresh weight and residual dry weight. The actual amounts are not given but only the percentages of the mean value at different times during the day and night. They smoothed the values actually obtained by taking the means of successive pairs of observations (6, p. 209). If we consider their curves derived from the residual-dry-weight data (Fig. 1, p. 209 and Fig. 2, p.

213 in Maskell and Mason's article) we find that the evidence in favor of diurnal change in total nitrogen, i.e., increases by day and decreases by night, is not entirely convincing. Thus, in their Figure 1 the loss from 6:30 p.m. to 10:30 p.m. is nearly balanced by the gain from 10:30 p.m. to 2:30 a.m., this latter change being one that is not understandable on the basis of a loss during darkness; in their Figure 2 the loss from 7:30 p.m. to 1:30 a.m. is followed by an unexpected rise from 1:30 a.m. to 7:30 a.m.; it is true that their Figure 1 shows a gain in nitrogen from 6:30 a.m. to 2:30 p.m. but in their Figure 2 the nitrogen in the leaves appeared to remain practically constant from 7:30 a.m. to 7:30 p.m. We thus find a lack of correlation in time between light and darkness on the one hand, and gains and losses in nitrogen on the other. The data are insufficient to establish satisfactorily the thesis that nitrogen increases in the leaf during the day and decreases during the night.

From this it should not be concluded that the position taken in the present paper is that no change in nitrogen takes place during the night. There is some evidence in the paper that there is a small loss. Of the 37 comparable pairs of tests the total nitrogen was lower in the morning in 22 and higher in 15 cases. The question is still open for further investigation. Perhaps there is a translocation of nitrogen from leaves under special conditions such as occur when fruits, seeds, or other storage organs are forming rapidly, or in woody plants during the period just preceding leaf fall. Possibly in such cases the rate of translocation is greater by night than by day.

The position taken is that the nitrogen metabolism is not comparable to that of carbohydrate. Diurnal change in carbohydrate is a common phenomenon in leaves of most plants, is usually large, and is easily demonstrated. No such situation had been proved with regard to nitrogenous substances. Perhaps a theory of diurnal change of nitrogenous substances would seem to be needed if one believes that protein synthesis takes place mainly in the leaves in the light and that such nitrogenous reserves must be translocated to cells not exposed to light. The need for such a theory disappears if we adopt the view that protein synthesis can occur in all active cells that have a supply of carbohydrates and simple nitrogenous substances whether the cells are in light or in darkness.

Starch. Even in cases in which the dry weight changes were small there were comparatively large percentage (not absolute) changes in the starch content of the leaves. This is shown particularly well by the data for peach and hawthorn in Table VII.

SUMMARY

1. Since it is believed that previous measurements of diurnal changes in leaves in which the computations were based upon the fresh weight or

TABLE VII
CHANGES IN LEAVES DURING THE NIGHT
RESIDUAL-DRY-WEIGHT-METHOD WITH WHOLE LEAVES

Name of plant	Time of sampling	Amount per gram of residual dry weight: Fresh wt. g.	Dry wt. g.	Sol. N. mg.	Insol. N. mg.	Poly-sacch. mg.	Starch mg.	Total sugar mg.	Per cent gain (+) or loss (−) during night compared with % difference between simultaneous samples: Fresh weight p.m. to a.m.	Fresh weight Si-mult.	Dry weight p.m. to a.m.	Dry weight Si-mult.	Total N. p.m. to a.m.	Total N. Si-mult.	Total carbohydrate p.m. to a.m.	Total carbohydrate Si-mult.
Tobacco	p.m.	7.64	1.196	5.20	48.5	167	133	28.2	+11.5	1.7	−10.4	0.3	+4.1	3.7	−64.0	3.8
		7.83	1.190	6.18	45.8	164	127	25.3								
	a.m.	8.66	1.068	5.34	48.2	53	13	14.3								
		8.59	1.069	5.98	50.5	56	12	13.9								
Sunflower	p.m.	8.22	1.165	4.78	52.8	141	68	23.8	+17.6	1.6	−8.3	1.0	+4.1	2.9	−56.2	7.3
		8.08	1.184	4.03	54.3	159	76	25.6								
	a.m.	9.65	1.075	4.19	54.8	72	8	3.2								
		9.50	1.078	4.31	57.4	73	11	5.2								
Sunflower	p.m.	7.00	1.155	2.08	44.9	140	71	15.6	+24.4	—	−6.0	—	−1.9	—	−44.4	—
	a.m.	8.71	1.086	2.17	43.9	79	6	7.5								
Cotton* (bolls on)	p.m.	5.78	1.149	4.37	39.1	124	61	24.8	+ 3.6	3.2	−2.9	0.3	−4.5	0.8	−22.2	3.1
		5.93	1.157	4.86	38.9	131	56	26.3								
	a.m.	5.95	1.119	4.59	36.9	97	31	21.8								
		6.18	1.119	4.58	37.3	98	32	19.5								
Cotton** (bolls off)	p.m.	5.38	1.272	4.58	39.5	235	162	36.4	+ 9.2	1.4	−9.1	0.3	+6.6	4.8	−42.5	1.6
		5.23	1.271	5.08	42.0	239	179	31.6								
	a.m.	5.82	1.153	4.84	43.1	137	70	16.0								
		5.82	1.159	5.52	43.8	143	71	16.3								

TABLE VII (*Continued*)
CHANGES IN LEAVES DURING THE NIGHT
RESIDUAL-DRY-WEIGHT-METHOD WITH WHOLE LEAVES

Name of plant	Time of sampling	Amount per gram of residual dry weight							Per cent gain (+) or loss (−) during night compared with % difference between simultaneous samples							
									Fresh weight		Dry weight		Total N.		Total carbohydrate	
		Fresh wt. g.	Dry wt. g.	Sol. N. mg.	Insol. N. mg.	Poly-sacch. mg.	Starch mg.	Total sugar mg.	p.m. to a.m.	Si-mult.	p.m. to a.m.	Si-mult.	p.m to a.m.	Si-mult.	p.m. to am..	Si-mult.
Grape	p.m.	3.28 3.28	1.148 1.146	8.15 8.25	28.9 27.3	122 120	41 45	25.7 25.3	+10.8	0.4	−2.5	0.5	−0.7	2.2	−19.3	4.7
	a.m. †	3.65 3.62	1.122 1.114	8.30 8.91	27.8 27.1	103 92	28 27	20.0 21.3								
Hawthorn	p.m.	2.52 2.49	1.129 1.122	1.14 1.02	30.3 29.6	110 104	25 27	18.9 18.0	+ 7.6	1.9	−1.1	0.7	+3.0	2.1	− 9.0	6.2
	a.m.	2.73 2.66	1.118 1.108	1.34 1.33	30.9 30.4	102 93	12 15	16.4 17.1								
Peach	p.m.	3.21 3.27	1.231 1.223	3.82 3.91	38.5 38.9	193 187	70 70	37.9 35.7	+10.9	1.0	−1.1	0.3	−1.6	0.9	− 5.7	2.0
	a.m. †	3.60 3.59	1.214 1.214	3.77 4.00	38.0 38.0	180 180	49 43	35.0 33.6								

* Leaves from plants of strain Super Seven; ** leaves from plants of different varieties equally distributed as to p.m. and a.m. samples.
† Rain during the night.

upon the leaf area basis are probably questionable because of the likelihood of changes in water content and the consequent increase or decrease in leaf area during the period of observation, experiments were undertaken by three other methods which make possible the elimination of errors in these respects.

2. The three methods used were: (1) the twin-leaf method by which the paired leaves of species having opposite leaves or leaflets are utilized to obtain uniform samples, one leaf of each pair being taken at the beginning of the experimental period and the other leaf of each pair at the end. The results may be expressed on the basis of the total amount of each constituent, and changes in water content, fresh weight, or leaf area do not interfere with an estimate of the changes during the period; (2) the half-leaf method which is similar to the twin-leaf method except that halves of leaves are cut off with a sharp knife along the midrib of the leaf, the other half of the leaf being left on for the subsequent sample. These results also may be calculated on the absolute basis, and percentages of constituents need not be dealt with; (3) the residual-dry-weight-method by which all constituents are estimated on the basis of the dry weight after subtracting the total carbohydrate from it. Evidence from the twin-leaf and half-leaf experiments is given to show that this residual dry weight does not change materially from night to morning.

3. Samples of leaves were collected in the evening and the corresponding samples the next morning. The tissues so collected were analyzed and data are given regarding the fresh weight, dry weight, soluble and insoluble nitrogen, alcohol-insoluble acid-hydrolyzable polysaccharide, starch, and total sugar. The plants used were tobacco, salvia, sunflower, hawthorn, redbud, beans, lilac, Virginia creeper, peach, soybean, cotton, and grape.

4. Corresponding to the night and morning samples was an approximately equal number of simultaneous samples, the object being to determine the extent of the sampling error so that the differences between the night and morning samples could be compared with the differences obtainable on samples collected simultaneously.

5. In nearly all experiments there was a gain in fresh weight during the night. This emphasizes the unsuitability of fresh weight as a basis for calculating diurnal changes in leaves. In cases in which there was no change in a constituent, calculation on the fresh weight basis would have indicated a loss during the night.

6. The dry weight decreased markedly with some species but this decrease was very small with others. A distinction was noted between the behavior of herbaceous types such as tobacco and sunflower and woody forms such as lilac and hawthorn. Herbaceous plants showed large changes from night to morning but with woody plants these changes were compara-

tively small. Thus, in lilac the measurements did not prove conclusively that there was any loss in the dry weight of lilac leaves during the night.

7. Changes in nitrogen were small. In 22 of the 37 comparable tests with different species, there was a loss and in 15 a gain in nitrogen during the night. But in most cases the differences between night and morning samples were not significantly greater than between simultaneous samples. Prolonging the period of darkness caused a breakdown of insoluble forms of nitrogen into soluble forms, but there was no translocation of nitrogen from the leaves. It is believed that the previously proposed theory that there is an analogy between the metabolism of nitrogenous substances and that of the carbohydrates has not been established conclusively at the present time.

8. The most pronounced change observed was that of the carbohydrates. In some species more than half of the total carbohydrates present in the leaves in the evening had disappeared during the night. Even larger percentage losses of starch were observed.

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RELATION OF ROOTING RESPONSE TO AGE OF TISSUE AT THE BASE OF GREENWOOD CUTTINGS

A. E. HITCHCOCK AND P. W. ZIMMERMAN

The success in rooting cuttings of shrubs and trees is dependent largely upon the age of the material used. A certain time of year is selected when the shoots are believed to be of the "proper degree of maturity." In this manner material is secured which is referred to as "softwood," "greenwood," "hardwood," "half-ripened wood," etc. There are many reports concerning the best time of year to take cuttings of woody plants. The experiments reported in this paper, however, deal mainly with the comparison in rooting of cuttings which were taken at the same time. Therefore, reference will be made only to those papers which report experiments of a similar nature.

Zimmerman (9, 10) has shown that if long greenwood canes of the American Pillar rose or of Weigela (*Diervilla*) are cut into three-inch segments, one cutting of such a series will root more readily than the others. Zimmerman (9) also showed that in young shoots of the Dorothy Perkins rose the roots grew readily from the basal portion whether or not a mallet piece of last season's wood was attached. For the American Pillar rose, however, the presence of a mallet piece practically inhibited the growth of roots; but when the mallet piece was removed, roots grew readily from the base of the young shoot. Ware (7) found that "simple cuttings" of the southern blueberry taken in April gave a higher percentage of rooting than either mallet or heel cuttings. Wyman (8) found no significant differences in the rooting of narrow-leaved evergreen cuttings with and without a heel, although he stated that large cuttings rooted better than small ones. Kemp (6) found that simple cuttings of the lilac (*Syringa vulgaris* var. Ludwig Spaeth) rooted better than heel cuttings and that cuttings taken at the internode were better than those taken at the node.

When maximum rooting is obtained at a particular time of year, it is difficult to tell whether such results are due entirely to the age of the shoot or to some definite portion of the cutting, such as the basal part, for example. Therefore, similar shoots with different aged wood at the base were used as cuttings and they were placed in the rooting medium on the same day. In a preliminary report (3) it was shown that the relation between rooting response and the age of the tissue at the base of the cutting varied with the species of plant. The present paper gives additional tests and a more complete classification of responses.

MATERIALS AND METHODS

Shoots three to five inches long of the current season's growth were made into the following four types of cuttings: (I) a mallet of last season's

wood, (II) a heel of last season's wood, (III) the cut made exactly at the base of the current season's growth, (IV) the cut made from one-fourth to three-fourths of an inch above the base of the current season's growth. Each of these types is illustrated in Figure 1. None of the terminal part of the shoot was removed, so that the difference in the age of the tissue was confined to the basal portion of the cutting. Since cuttings of most species were made from relatively slow growing shoots, type IV cutting is not necessarily the same as the "top cutting" or "simple cutting" of fairly active material commonly used by propagators.

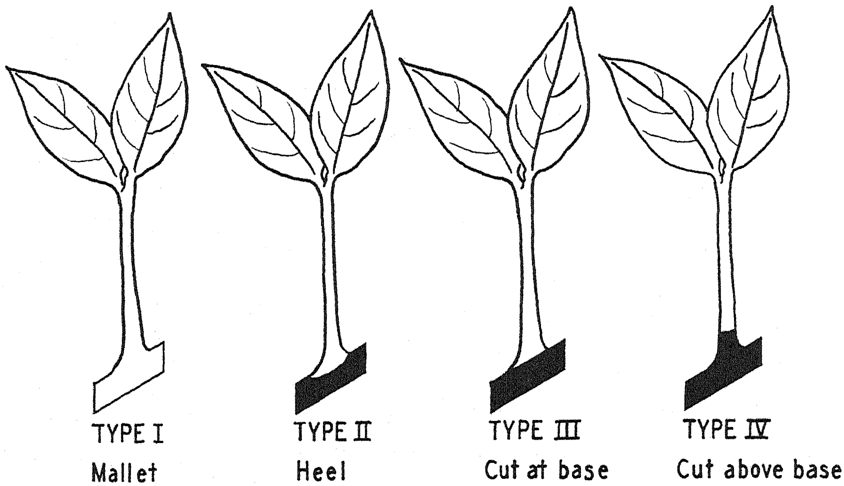

FIGURE 1. Four types of cuttings made from similar shoots. The portion eliminated from the base of the cutting for types II, III, and IV is shown in black.

Most of the cuttings were rooted in sand in greenhouse benches. The blueberry (*Vaccinium corymbosum*) cuttings, however, were planted in a mixture of half sand and half peat moss by volume. The sand purchased from the Yonkers Builders Supply Company came originally from Cow Bay, Long Island. The granulated peat moss was obtained from Atkins and Durbrow, Inc., New York City. Cutting material was obtained from plants growing on the property of the Boyce Thompson Institute. Practically all cuttings were taken during the period May 9 to August 20. Ten cuttings were used for each test. Extra cuttings of each type were placed in the rooting medium for observational purposes. The condition of these cuttings at various times determined when the experimental lots were to be taken out.

The cuttings were placed *deeply* in sand or in a mixture of peat moss and sand so that only a few leaves were exposed (Fig. 2). Some or all of the leaves were left on the portion of the stem which was buried. A con-

siderable amount of water was added to settle the sand around the cuttings. While adding the water, the tops of the cuttings were pressed down with the palm of the hand, as a result of which most of the exposed leaves thereafter lay close to or nearly flat on the surface of the rooting medium. A single layer of cheesecloth was laid on the tops of the cuttings. Subsequent applications of water were made over the cloth. During cloudy periods or in cool weather the cheesecloth was removed, but it usually remained on the cuttings overnight during the spring and summer months. The cloth was sprayed several times a day during hot weather. No sash was used to protect cuttings outside in frames or in the greenhouse. The

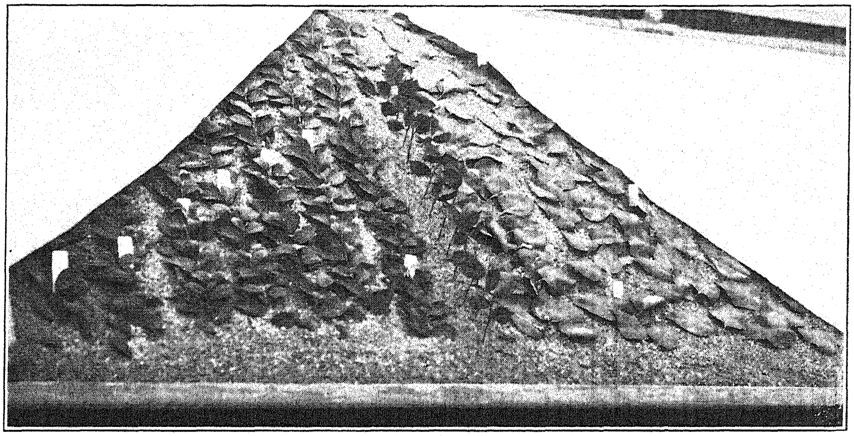

FIGURE 2. Section of a propagating bench showing the method of planting softwood cuttings. The cuttings, which have few or no leaves removed, are placed deeply in the rooting medium and are then covered with a single layer of cheesecloth. Cuttings of *Prunus tomentosa* are shown at the left and lilac cuttings at the right. At the time of photographing a few cuttings with the lower leaves stripped off were placed in an upright position merely to contrast the method just described with the one commonly used by propagators.

southern slope of the greenhouse was provided with a slat shade which could be rolled up during cloudy weather. Although a relatively high humidity was maintained close to the cuttings, the atmosphere above them could have been relatively dry without causing detrimental effects. Cuttings handled by this method do not require the critical care, particularly in regard to watering and ventilation, that those do which are handled under sash according to the standard commercial practice.

EXPERIMENTAL RESULTS

The irregular distribution of the values in Table I for the average percentage of cuttings rooted and the average size of the root system shows that rooting response varied with the type of cutting and with the species

TABLE I

CLASSIFICATION OF ROOTING RESPONSE ACCORDING TO THE TYPE OF CUTTING

Group No.	Plant	No. tests made	Rooting response							
			Type I Mallet		Type II Heel		Type III Cut at base		Type IV Cut above base	
Group 1 Good rooting for all types of cuttings	*Callicarpa japonica* Thunb	1	100*	A†	100	A	100	A	100	A
	Physocarpus opulifolius Maxim. var. *intermedius* (Rybd.) Robinson	2	100	A	85	A	100	A	100	A
	Physocarpus opulifolius Maxim.	2	100	A	100	A	100	A	100	A
	Spiraea bumalda Burv. var. Anthony Waterer	2	100	A	100	A	100	A	100	A
	Viburnum cassinoides L.	2	100	A	95	A	—	—	95	A
	Viburnum opulus L., var. *americanum* (Mill.) Ait.	6	93	B	96	B	100	B	98	B
Group 2 Two or three types of cuttings superior to others	*Callicarpa purpurea* Juss.	2	65	B	100	A	100	A	100	A
	Forsythia intermedia Zabel.	9	78	C	99	A	97	A	97	A
	Forsythia suspensa Vahl.	4	90	C	100	A	—	—	100	A
	Forsythia viridissima Lindl.	5	77	B	100	A	93	A	70	B
	Philadelphus falconeri Sarg.	2	90	B	100	A	100	A	100	A
	Philadelphus sp. (kalerani)	2	80	C	95	C	90	C	80	C
	Philadelphus magdalenae Koehne.	2	60	C	95	C	85	C	70	C
	Philadelphus pubescens Loisel.	1	100	A	100	A	90	A	90	A
	Rosa (Dorothy Perkins)	2	100	A	100	A	—	—	40	C
	Rosa omeiensis Rolfe.	4	0	C	55	C	55	C	35	C
	Rosa setigera Michx.	2	100	A	100	A	90	A	30	C
	Spiraea salicifolia L.	2	95	A	90	A	90	B	90	B
	Symphoricarpos orbiculatus Moench.	2	95	A	90	A	90	B	100	A
	Vaccinium corymbosum L.	4	30	C	90	B	85	A	65	B
	Viburnum pubescens (Ait.) Pursh.	2	70	C	100	A	95	A	85	B

TABLE I (*Continued*)

CLASSIFICATION OF ROOTING RESPONSE ACCORDING TO THE TYPE OF CUTTING

Group No.	Plant	No. tests made	Rooting response							
			Type I Mallet		Type II Heel		Type III Cut at base		Type IV Cut above base	
Group 3 One type of cutting superior to other three	*Berberis thunbergii* DC.	4	28	C	26	C	3	C	68	B
	Cornus florida L.	12	80	A	80	C	—	—	30	C
	Cornus paniculata L'Her.	4	35	C	80	B	53	C	68	B
	Ligustrum ovalifolium Hassk.	6	65	C	45	C	100	A	90	A
	Lonicera morrowi Gray.	6	40	C	70	B	81	B	65	B
	Lonicera tatarica L.	3	67	B	73	B	90	A	73	B
	Prunus glandulosa Thunb.	6	53	C	44	C	80	A	57	B
	Prunus tomentosa Thunb.	11	35	C	12	C	60	C	82	B
	Prunus (Darrow's special)	1	10	C	10	C	—	—	80	B
	Rosa (American Pillar)	2	55	C	90	A	100	A	90	C
	Rosa hugonis Hemsl.	7	53	C	97	A	80	A	54	C
	Spiraea vanhouttei Zabel.	4	40	C	85	C	89	B	95	A
	Syringa vulgaris L.	7	28	C	70	B	84	B	54	C
	Ulmus pumila L.	6	56	C	55	C	50	C	82	B
	Vaccinium corymbosum L. var. Adams	5	35	C	63	B	72	B	62	C
	Vaccinium corymbosum L. var. Rubel	5	21	C	64	C	73	B	85	A
	Vaccinium corymbosum L. var. Pioneer	2	15	C	90	C	80	B	75	B
	Diervilla hybrida Dipp.	3	93	B	60	C	80	B	53	C

* Numerical values represent the average percentage of rooting.

† Letters denote the ratings for the average size of the root systems, as follows: A, 80 to 100 per cent of the cuttings with good roots; B, 50 to 70 per cent of the cuttings with good roots; C, less than 50 per cent of the cuttings with good roots.

TABLE II

CLASSIFICATION OF PLANTS ACCORDING TO THE RELATIVE ROOTING FOR THE FOUR TYPES OF CUTTINGS*

Type of cutting	Group 1 Good rooting for all types of cuttings	Group 2 Good rooting for two or three types of cuttings	Group 3 One type of cutting superior to the other three
I Mallet	*Callicarpa japonica* *Physocarpus opulifolius* var. *intermedius* *Physocarpus opulifolius* *Spiraea bumalda* var. Anthony Waterer *Viburnum cassinoides* *Viburnum opulus* var. *americanum*	*Philadelphus pubescens* *Rosa* (Dorothy Perkins) *Rosa setigera* *Spiraea salicifolia* *Symphoricarpos orbiculatus*	*Cornus florida* *Diervilla hybrida*
II Heel	*Callicarpa japonica* *Physocarpus opulifolius* var. *intermedius* *Physocarpus opulifolius* *Spiraea bumalda* var. Anthony Waterer *Viburnum cassinoides* *Viburnum opulus* var. *americanum*	*Callicarpa purpurea* *Forsythia intermedia* *Forsythia suspensa* *Forsythia viridissima* *Philadelphus falconeri* *Philadelphus* (kalerani) *Philadelphus magdalenae* *Philadelphus pubescens* *Rosa* (Dorothy Perkins) *Rosa omeiensis* *Rosa setigera* *Spiraea salicifolia* *Symphoricarpos orbiculatus* *Viburnum pubescens*	*Cornus paniculata* *Rosa hugonis*

TABLE II (*Continued*)

CLASSIFICATION OF PLANTS ACCORDING TO THE RELATIVE ROOTING FOR THE FOUR TYPES OF CUTTINGS*

Type of cutting	Group 1 Good rooting for all types of cuttings	Group 2 Good rooting for two or three types of cuttings	Group 3 One type of cutting superior to the other three
III Cut at base	*Callicarpa japonica* *Physocarpus opulifolius* var. *intermedius* *Physocarpus opulifolius* *Spiraea bumalda* var. Anthony Waterer *Viburnum opulus* var. *americanum*	*Callicarpa purpurea* *Forsythia intermedia* *Forsythia viridissima* *Philadelphus falconeri* *Philadelphus* (kalerani) *Philadelphus magdalenae* *Rosa* (Dorothy Perkins) *Rosa omeiensis* *Rosa setigera* *Vaccinium corymbosum* (Pioneer) *Viburnum pubescens*	*Ligustrum ovalifolium* *Lonicera morrowi* *Lonicera tatarica* *Prunus glandulosa* *Rosa* (American Pillar) *Syringa vulgaris* *Vaccinium corymbosum* *Vaccinium corymbosum* (Adams)
IV Cut above base	*Callicarpa japonica* *Physocarpus opulifolius* var. *intermedius* *Physocarpus opulifolius* *Spiraea bumalda* var. Anthony Waterer *Viburnum cassinoides* *Viburnum opulus* var. *americanum*	*Callicarpa purpurea* *Forsythia intermedia* *Forsythia suspensa* *Philadelphus falconeri* *Symphoricarpos orbiculatus* *Vaccinium corymbosum* (Pioneer)	*Berberis* *Prunus tomentosa* *Spiraea vanhouttei* *Ulmus pumila* *Vaccinium corymbosum* (Rubel)

* Rating values for the rooting of all cuttings appear in Table I.

of plants. In Table I the letters A, B, and C represent the average size or rating of the roots as excellent, fair, and poor respectively. Therefore it is necessary in evaluating the effectiveness of each type of cutting to consider both the percentage rooted and the ratings indicated by letters. However, when the species are arranged according to the relative effectiveness of each type of cutting in producing good roots, they fall into three main groups as shown in Table II.

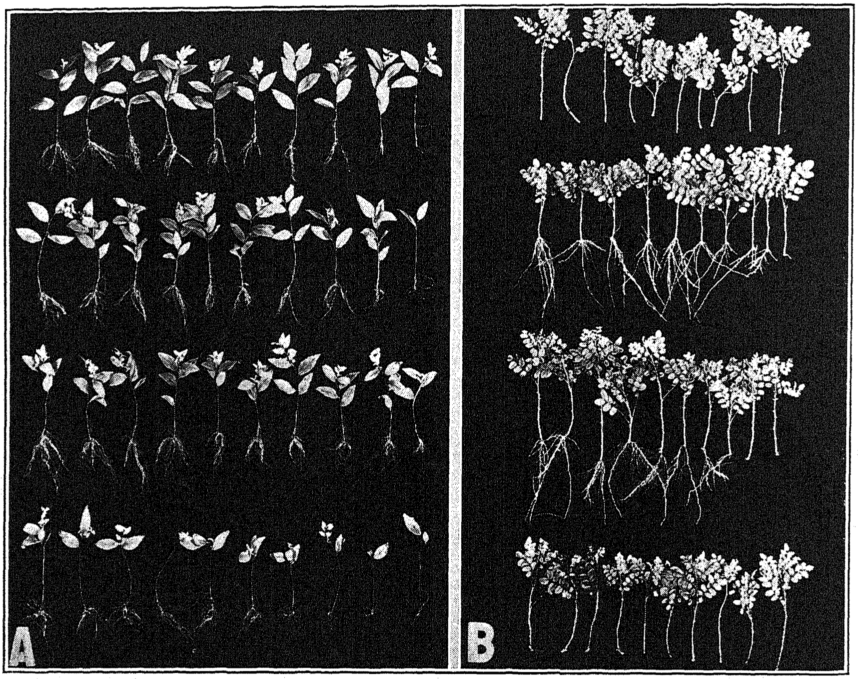

FIGURE 3. The difference in rooting of (A) *Spiraea bumalda* and (B) *Rosa hugonis* from four types of softwood cuttings arranged from top to bottom as follows: type IV (cut made above base), type III (cut made at base), type II (a heel of last season's wood), and type I (a mallet of last season's wood).

Group 1 (Table II) includes species which showed no marked difference in rooting response for the four types of cuttings (Fig. 3, A). For the species in group 2 there were two or three types, but not necessarily the same ones, which produced good roots (Fig. 3, B). In group 3 are the species for which one type of cutting was better than the other three (Fig. 4, A and B). These data show that type III cuttings produced good rooting for the largest number of species and type I cutting produced good roots for the fewest number of species.

Cornus florida and *Diervilla hybrida* (Weigela) are the only species

listed in Table II opposite "Type I Mallet" which fail to appear opposite the other types. In only these two cases was the mallet piece found to be essential for good rooting. Another type of cutting produced rooting comparable to the mallet for 11 species and better than the mallet for 25 species. Roots grew from the mallet piece, from the stem above the mallet piece, or from both places. Certain species which rooted with difficulty from mallet cuttings also rooted with difficulty from hardwood cuttings. This was true of *Vaccinium*, *Syringa*, *Prunus*, *Lonicera*, *Forsythia*, *Rosa*

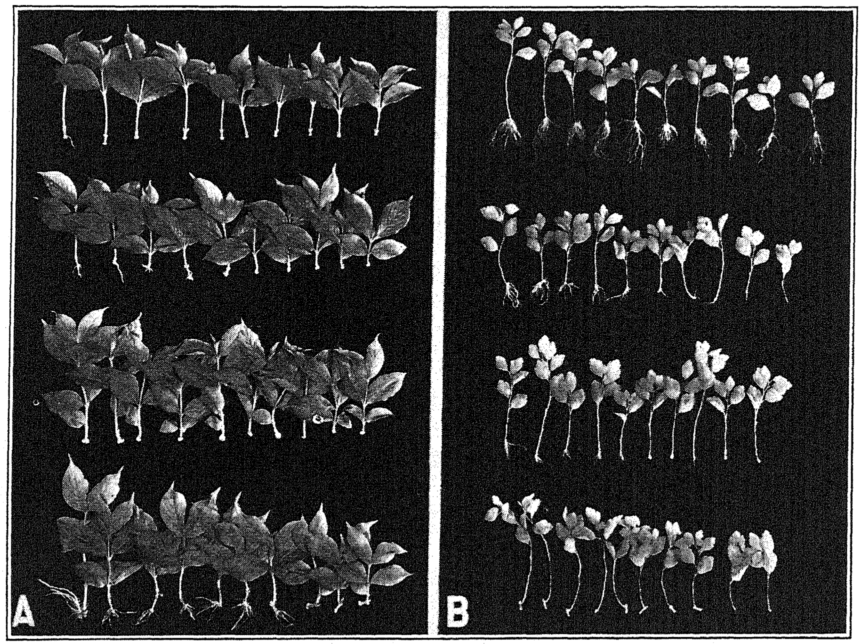

FIGURE 4. The difference in rooting of (A) *Diervilla hybrida* and (B) *Spiraea vanhouttei* from four types of softwood cuttings arranged from top to bottom as follows: type IV (cut made above base), type III (cut made at base), type II (a heel of last season's wood), and type I (a mallet of last season's wood).

hugonis, *Rosa omeiensis*, and Pillar rose. Such a correlation has not been determined for all of the species given in Table I and it may be that exceptions occur.

The reason why a mallet piece retards rooting in some species and not in others has not been worked out. It has been observed, however, that mallet cuttings of certain species showed wilting more readily than the other three types of cuttings. Such a response might be due to an interference with the intake of water by the mallet piece.

Since the type III cutting was the best for most of the species of plants,

the question arises as to why this type is not more generally used. Probably one reason is that the time at which cuttings of certain plants must be taken is governed by a routine that often prevents the collection of material when the shoots are of the proper length. Also, there is a belief among propagators that the basal cut should be made in a definite relation to the bud or node, in which case the cut would have to be made above the point designated for a type III cutting.

Species or varieties of certain genera did not respond alike. In the case

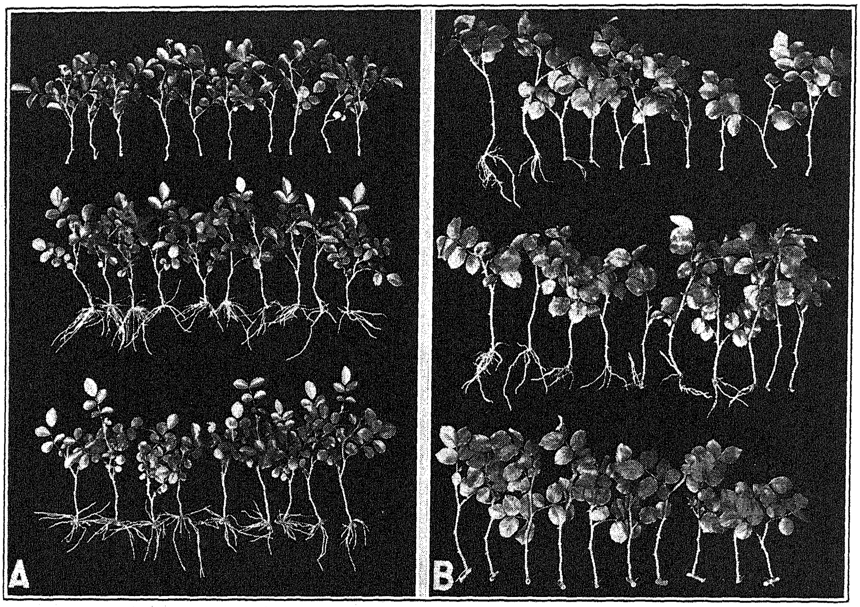

FIGURE 5. The difference in rooting of (A) Dorothy Perkins rose and (B) American Pillar rose from three types of cuttings arranged from top to bottom as follows: type IV (cut made above base), type II (a heel of last season's wood), and type I (a mallet of last season's wood).

of Dorothy Perkins rose cuttings, roots grew readily from the base of the current season's growth, whether or not a mallet piece was present (Fig. 5, A). For similar cuttings of the American Pillar rose, root growth was practically prevented by the presence of a mallet piece (Fig. 5, B). Type IV cuttings rooted poorly, showing that the basal portion of the young shoot is an important region for root production in both roses. According to Carlson (2) there is formed at the base of Dorothy Perkins shoots a special pad of secondary phloem cells from which the roots emerge. Similar tissue was not formed at the base of Pillar shoots, yet when the mallet piece is removed from Pillar cuttings, rooting takes place readily. It is in-

teresting that such a dissimilar response would occur for two hybrids which have a common parent, namely, *Rosa wichuraiana*. *Rosa setigera*, the other parent of the Pillar rose, responded like Dorothy Perkins. Removal of the base of the young shoot retarded the rooting not only of the three closely related roses just mentioned, but also of the species *Rosa hugonis* (Fig. 3, B) and *Rosa omeiensis*. The latter two species responded similarly to the Pillar rose from all four types of cuttings.

Mallet cuttings of *Spiraea bumalda* and *S. salicifolia* were easily rooted,

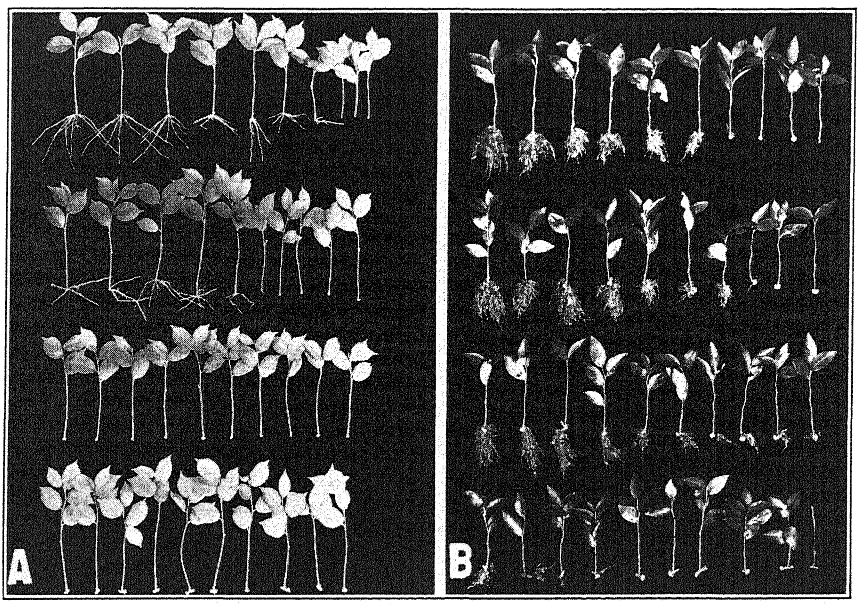

FIGURE 6. The difference in rooting of (A) *Prunus tomentosa* and (B) *Vaccinium corymbosum* var. Adams from four types of softwood cuttings arranged from top to bottom as follows: type IV (cut made above base), type III (cut made at base), type II (a heel of last season's wood), and type I (a mallet of last season's wood).

but all of the roots did not emerge from the base of the shoot. On the other hand, *S. vanhouttei* rooted poorly from mallet cuttings but showed strong basal polarity in rooting. *Philadelphus pubescens* rooted exceptionally well from mallet cuttings, but this was not true for the other three species of *Philadelphus*.

The wild blueberry (*Vaccinium corymbosum*) and the cultivated varieties Adams, Pioneer, and Rubel were easily rooted from all types except the mallet cutting. These data are in agreement with Ware's (6) results for the southern blueberry in which it was shown that simple cuttings (comparable to type IV described in this paper) gave a higher percentage

of rooting than mallet cuttings. In our experiments the variety Rubel rooted best from type IV cuttings taken in July, but for cuttings taken in August types II and III were equally as good. Similar differences for the variety Adams were observed in comparing the response of the July and August lots. Although the lot of Adams cuttings shown in Figure 6, B rooted best from heel cuttings, the average values for the five lots taken in July and August showed that type III cutting was best from the standpoint both of the percentage of rooting and the size of the root systems. All four varieties of *Vaccinium* showed marked basal rooting.

According to Beckwith and Coville (1) hardwood cuttings taken in late winter are the chief source of new blueberry plants for the commercial growers, since no satisfactory method has been established for softwood cuttings. While Johnston (4) was able to root softwood cuttings of the cultivated varieties in German peat moss, he obtained less than 10 per cent rooting for the cuttings placed in a mixture of peat moss and sand. Johnston (5) later stated, "The rooting of blueberry cuttings requires considerable equipment, a comparatively long time, and more or less constant attention." Ware (6), on the other hand, obtained a high percentage of rooting for cuttings placed either in pure peat moss or in a mixture of peat moss and sand, although he recommends the latter medium. No doubt his success with the southern blueberry would hold for the cultivated varieties. Our results show that the blueberry (*Vaccinium corymbosum*) may be rooted with comparative ease from softwood cuttings placed in a mixture of peat moss and sand, for all types of cuttings except the mallet. Placing the cuttings, from which few or no leaves have been removed, deeply in the medium is regarded as an important procedure in handling softwood cuttings of the blueberry as well as of many other species, since with this method no sash is required either in the greenhouse or outside in frames.

While the main purpose of these experiments was to determine the relative difference in response for the four types of cuttings, certain seasonal differences were observed. Mallet cuttings gave a uniformly poor response for all lots of *Vaccinium*, but the other three types of cuttings varied in response when they were taken at two different times. Mallet cuttings of *Diervilla* (Fig. 3, A) and of *Cornus florida* taken in May rooted much more readily than the other three types, but when taken after June 15, all four types were easily rooted. However, mallet cuttings were uniformly poor during the summer for the following species: *Forsythia intermedia* and *F. suspensa*, the three species of *Prunus*, *Spiraea vanhouttei*, and *Syringa vulgaris*.

Lilac shoots taken in May rooted readily from type II (heel) and type III (cut at base) cuttings, but the rooting of type I (mallet) and type IV (cut above base) was relatively poor. Apparently the common lilac does

not respond the same as the variety Ludwig Spaeth used by Kemp (6), since he found that simple cuttings of this variety rooted better than heel cuttings. Kemp's cuttings were taken in June and July and they were left in the rooting medium until November, whereas our cuttings were taken on May 12 and removed after 21 to 32 days. In one lot, 80 per cent of the type III cuttings rooted in 21 days. The rooting response of the common lilac, for all types except the mallet cutting, depended mainly upon the time of year the material was taken. Cuttings made from young shoots in which the buds were active, rooted readily and thereafter produced a vigorous top growth the same season. If the cuttings were taken later, particularly after June 15, at a time when the buds had become dormant, rooting was slower and there was no top growth. The failure of cuttings taken in June or thereafter to make a satisfactory growth the same season is probably the reason why commercial growers have resorted to grafting the lilac instead of propagating it from cuttings.

These data show that in addition to a knowledge of the best time to take cuttings, it is important to know the type of cutting which will give maximum rooting at any particular time. Although species varied considerably as to the type of cutting which gave the best rooting, the mallet type was generally poor and type III (cut at base) was usually good.

SUMMARY

1. The rooting response of greenwood material was compared for the following four types of cuttings: (I) a mallet of last season's wood, (II) a heel of last season's wood, (III) the cut made at the base of the current season's growth, (IV) the cut made from one-fourth to three-fourths of an inch above the base of the current season's growth.

2. Rooting varied with the type of cutting and with the species of plants.

3. Although no one of the four types of cuttings produced the best rooting for all species, the mallet type was generally poor and type III was usually good.

4. In certain cases different species of the same genus and different varieties of the same species showed differences in rooting.

5. The wild blueberry (*Vaccinium corymbosum*) and the cultivated varieties Adams, Pioneer, and Rubel rooted poorly from mallet cuttings, but they were easily rooted from the other three types of cuttings.

6. The common lilac was rooted readily in May from type II and type III cuttings and produced thereafter a vigorous top growth the same season.

7. A detailed description is given for a method of planting and protecting the cuttings without the use of sash.

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ESTIMATION OF SULPHYDRYL IN TISSUES[1]

JOHN D. GUTHRIE AND FRANK WILCOXON

The generally accepted method for determining the sulphydryl content of tissues is that of Tunnicliffe (11). It has been widely used both in its original and modified forms. This method is based on the reaction of sulphydryl compounds with iodine in trichloracetic acid solution and involves the assumption that sulphydryl compounds compose at least a major part of the substances in tissues that reduce iodine in acid solution. Hexuronic acid, a non-sulphydryl compound that reacts with iodine in acid solution, has been isolated from oranges, cabbage, and the adrenal cortex by Szent-Györgyi (10). It is probable that such non-sulphydryl iodine-reducing compounds are present in many tissues. Extracts of many plant tissues react with appreciable quantities of iodine, although the nitroprusside test is very slight or negative, indicating that the iodine reduction method is inapplicable. It will be shown that it is possible to separate the iodine-reducing substances of the potato into two fractions, one of which gives no nitroprusside test, thus showing definitely the presence of non-sulphydryl iodine-reducing substances and indicating that the iodine method will not give a true measure of the sulphydryl content of extracts of potato tissue.

Since the iodine method fails to measure the sulphydryl content of certain tissues, it was decided to investigate the reaction of sulphydryl compounds and tissue extracts with sulphur. It has long been known that sulphydryl compounds react with sulphur to form hydrogen sulphide and that this reaction is shown by many tissues. De Rey-Pailhade (8) in 1888 observed that alcoholic extracts of yeast produced hydrogen sulphide from sulphur. Heffter (4) suggested in 1908 that this reaction was due to the presence of sulphydryl compounds. In 1921 Hopkins (5) isolated glutathione from yeast. He mentions that it reacts with sulphur to form hydrogen sulphide. The only detailed study of this reaction since the isolation of glutathione is that of Sluiter (9). She studied the hydrogen sulphide production from minced animal tissues and glutathione solutions. Her results with tissues showed no definite relationship between the content of glutathione and the amount of hydrogen sulphide produced. Her yields of hydrogen sulphide from glutathione solutions were low, usually less than 25 per cent of the theoretical yield calculated from the reaction:

$$2\,RSH + S \rightarrow RSSR + H_2S$$

Sluiter's main conclusion is that the formation of hydrogen sulphide by the action of tissues on sulphur is not due to enzymes.

[1] Herman Frasch Foundation for Research in Agricultural Chemistry, Paper No. 33.

The reduction of sulphur is more specific for sulphydryl compounds than the reduction of iodine. The authors have not yet found a non-sulphydryl compound likely to be present in tissues that reacts with sulphur to form hydrogen sulphide. In order to determine the general applicability of this reaction, a study was made of the yield of hydrogen sulphide from cysteine, glutathione, and extracts of various tissues. The yield of hydrogen sulphide from tissue extracts to which known amounts of glutathione had been added was investigated. The sulphur reduction method was also tested by applying it to the iodine-reducing substances of the potato after they had been fractionated with lead acetate.

DEVELOPMENT OF THE METHOD

In preliminary experiments the lead acetate paper method used by McCallan and Wilcoxon (6) in their study of the formation of hydrogen sulphide by the action of fungous spores on sulphur was employed. Using this method, it was found that the oxidation of hydrogen sulphide by the air is catalyzed by sulphur. Therefore, it was decided to reduce the amount of sulphur used by adding it in alcoholic solution. It was also found that phosphate buffer tended to protect hydrogen sulphide from oxidation by the air in the presence of sulphur and therefore a phosphate buffer was used to regulate the hydrogen ion concentration. The lead paper method is extremely sensitive and capable of detecting very small amounts of hydrogen sulphide. In the present work, however, the amount of the gas evolved permitted the use of the more accurate method of Almy (1) which depends on the absorption of the hydrogen sulphide in zinc acetate solution and its conversion to methylene blue by the addition of para-amino-dimethylaniline and ferric chloride. It was found necessary to increase the amounts of para-amino-dimethylaniline and ferric chloride above those recommended by Almy, in order to deal with the larger amounts of hydrogen sulphide encountered in these experiments. When this was done the methylene blue color became proportional to the sulphide concentration. Experiments were undertaken with cysteine hydrochloride and also with glutathione. It was found that the best results were obtained when the hydrogen sulphide was aerated rapidly with nitrogen from the reacting mixture into the zinc acetate. The methylene blue must be developed in the receiving tube, since the zinc sulphide formed could not be washed quantitatively from the receiver. Irregular results were obtained until this point was noted.

In deciding on a method for preparing the tissue extracts, it was obvious that destruction of sulphydryl compounds during the process of preparation must be avoided. These compounds are easily destroyed by oxidation, especially in the presence of oxidase. The inhibiting action of oxidase on the production of hydrogen sulphide has been reported in a previous paper (3). It was decided, therefore, to kill the tissue by dropping

into boiling water prior to the extraction. The necessity for doing this is well brought out by the fact that juice of untreated potato tubers, even if boiled to destroy oxidase, will not form hydrogen sulphide from sulphur. Since it was unlikely that potato tubers were entirely lacking in sulphydryl compounds, it was evident that the sulphydryl compounds present in the tissue were destroyed during the process of obtaining the juice. When the method of dropping the tissue into boiling water was tried, the extract was found to react with sulphur to form hydrogen sulphide.

Experiments undertaken to establish the time of aeration necessary for the completion of the reaction showed that almost all the hydrogen sulphide was obtained during the first two hours, and that aerating for three or four hours did not significantly increase the yield. This was true with both cysteine solutions and potato extracts. Three hours was chosen as the aeration time.

Experiments to determine the pH at which to carry out the reaction showed little change in yield between pH 6.4 and pH 7.3. It was decided to use a phosphate buffer having a pH of 6.8.

THE METHOD

Reagents required:

Phosphate buffer, pH 6.8. Prepare this by mixing equal volumes of M/15 $Na_2HPO_4.12H_2O$ and M/15 KH_2PO_4.

Zinc acetate, 6 g. per liter.

Saturated solution of sulphur in absolute alcohol. Prepare this by making up in hot absolute alcohol, allowing to cool and filtering.

Para-amino-dimethylaniline hydrochloride. Dissolve 100 mg. in 100 cc. of hydrochloric acid made by diluting the concentrated reagent with an equal volume of water.

Ferric chloride. Prepare this by dissolving 27 g. of $FeCl_3.6H_2O$ in 500 cc. of concentrated HCl and diluting to one liter. This makes a M/10 solution. Prepare a M/50 solution by dilution.

Other reagents needed are paraffin oil, 95 per cent alcohol, N/50 iodine, N/100 $Na_2S_2O_3$, and sodium hydrosulphide.

The procedure is as follows:

Cut the sample into pieces of about one gram each, and drop a weighed amount, usually 50 g., into 50 cc. of boiling water. Boil gently for 12 minutes. Cool by placing the beaker in cold water. Grind in a large mortar. Add 110 cc. of 95 per cent alcohol, make up with water to a volume of 200 cc. and mix. Centrifuge.

Place an aliquot portion of the supernatant liquid, usually 50 cc. into a Van Slyke-Cullen (12) aeration tube. If less than 50 cc. are used, add 50 per cent alcohol to make 50 cc. Add 10 cc. of phosphate buffer of pH 6.8, and 10 drops of paraffin oil. Place 20 cc. of 0.6 per cent zinc acetate in a

second aeration tube and connect it as a receiver to the first tube. Add 1 cc. of a saturated solution of sulphur in absolute alcohol to the first tube and aerate rapidly with nitrogen for three hours in order to drive over all the hydrogen sulphide into the receiving tube.

Develop methylene blue *in the receiving tube* by adding 5 cc. of the para-amino-dimethylaniline solution and 5 cc. of M/50 ferric chloride. After the color has developed for one hour or longer, make up to volume, usually to 50 cc. in a volumetric flask and compare in a colorimeter with the standard.

Prepare the standard by adding a known amount of NaSH to 20 cc. of zinc acetate and develop the color in the same manner as employed for the sample. A convenient method is to make up a solution of sodium hydrosulphide approximately N/100, standardize by pipetting into a known volume of N/50 iodine, and titrate the excess of iodine with N/100 $Na_2S_2O_3$. Prepare a N/1000 NaSH solution by diluting this solution. Five cc. of the N/1000 solution added to zinc acetate and made up to 50 cc., after developing the color, makes a convenient standard. The NaSH solutions are not stable and should be prepared just before using.

APPLICATION OF THE METHOD TO SOLUTIONS OF GLUTATHIONE AND CYSTEINE

In order to determine what fraction of the theoretical value could be recovered when using known amounts of cysteine and glutathione, and to find out whether the amount of hydrogen sulphide produced is proportional to the sulphydryl concentration, the method was applied to solutions containing known amounts of these compounds. The results are shown in Table I. Amounts are expressed in terms of milli-equivalents,

TABLE I

THE APPLICATION OF THE SULPHUR REDUCTION METHOD TO KNOWN AMOUNTS OF GLUTATHIONE AND CYSTEINE

Sulphydryl compound	Milli-equiv. of SH used	Milli-equiv. of H_2S recovered				Av. per cent recovery
		Exp. 1	Exp. 2	Exp. 3	Av.	
Glutathione	.0050	.0052	.0042	.0046	.0047	94
"	.0100	.0095	.0097	.0101	.0098	98
"	.0200	.0227	.0189	—	.0208	104
Cysteine	.0025	.0016	.0014	.0018	.0016	64
"	.0050	.0033	.0030	.0035	.0033	66
"	.0100	.0067	.0061	.0067	.0065	65
"	.0200	.0148	.0130	—	.0139	69

namely, 0.307 g. for glutathione and 0.017 g. for hydrogen sulphide. The amounts of sulphydryl are calculated on the basis of an assumed 100 per cent purity of the samples of glutathione and cysteine hydrochloride used. Iodine titrations using sodium nitroprusside as an external indicator

showed the purity of the cysteine hydrochloride sample to be 94 per cent and the purity of the glutathione sample to be 95 per cent. The nitroprusside method probably gives slightly low values. It will be seen in Table I that practically theoretical recovery of hydrogen sulphide was obtained in the case of glutathione, but only 65 per cent in the case of cysteine. The amount of hydrogen sulphide recovered was, however, proportional to the amount of cysteine used.

APPLICATION OF THE METHOD TO VARIOUS TISSUES

In order to investigate the usefulness of the sulphur reduction method and to compare it with the iodine reduction method, both were applied to extracts of various tissues. The iodine titration was applied according to the procedure of Perlzweig and Delrue (7). The results are shown in Table II. In some cases a known amount of glutathione was added to the extracts after the evolution of hydrogen sulphide by the sulphur reduction

TABLE II

APPLICATION OF THE SULPHUR REDUCTION METHOD TO EXTRACTS OF VARIOUS TISSUES AS COMPARED WITH THE IODINE REDUCTION METHOD

Tissue used	Cc. extract used	Milli-equiv. SH by		Per cent recovery of added GSH from same extracts	SH values by S reduction corrected for recovery	Ratio SH by S red. to SH by I red.
		Iodine reduction	Sulphur reduction			
Potato tubers (*Solanum tuberosum* L.)	50	.022	.0034	76	.0045	0.20
" leaves " "	50	.063	.0022	51	.0043	0.07
Gladiolus corms (*Gladiolus* sp. var. "Souvenir")	50	.068	.0032	87	.0037	0.05
Lilac buds (*Syringa vulgaris* L.)	50	.106	.0025	—	—	—
Yeast* (*Saccharomyces* sp.†)	5	.0041	.0035	91	.0038	0.93
"	10	.0082	.0077	91	.0085	1.04
Calves liver	10	.016	.0035	72	.0049	0.31
" "	25	.039	.0088	76	.0116	0.30

* A 25 g. sample was used in the case of yeast. Fifty gram samples were used with all other tissues.

† Fleischman's compressed yeast.

method had been completed, and the recovery from these extracts determined by adding more sulphur and aerating into fresh zinc acetate. The percentage recovery determined in this way is shown in column 5, Table II. Inspection of the data of Table II shows that the recovery of added glutathione as shown by the hydrogen sulphide formed is substantially complete only in the case of yeast, although, as shown previously, complete recovery can be obtained with pure glutathione solutions. Even after correcting the values obtained by the sulphur method for recovery, they are much lower than the values obtained by the iodine method, except in the case of yeast. It is believed that the large discrepancies in the case of

the other tissues are due to the presence of non-sulphydryl iodine-reducing substances. Evidence of the simultaneous presence of sulphydryl and non-sulphydryl iodine-reducing compounds in the potato is given by the following experiment.

FRACTIONATION OF THE IODINE-REDUCING SUBSTANCES OF THE POTATO

Juice of untreated tubers and of tubers treated with 40 cc. of 40 per cent ethylene chlorhydrin by the dip method of Denny (2) was obtained five days after treatment. The juice from each sample was centrifuged, boiled, and the coägulum removed by centrifuging. To 50 cc. of the boiled juice, 20 cc. of saturated lead acetate were added; the precipitate was separated by centrifuging and washed with lead acetate diluted 1 to 4. A second portion of saturated lead acetate, 10 cc., was added to the combined washings and supernatant liquid, and the precipitate centrifuged down and washed with saturated lead acetate. The two precipitates were combined and deleaded by adding 10 cc. of 10 per cent sulphuric acid and centrifuging out the lead sulphate. This precipitate was washed with water, the supernatant liquid and washings carefully neutralized with 20 per cent sodium hydroxide, using methyl red as an indicator, and made up to 50 cc. This will be referred to as fraction No. 1.

The combined washings and supernatant liquid from the first and second lead acetate precipitations were made alkaline with 5 cc. of concentrated ammonium hydroxide. The precipitate was centrifuged out, deleaded, and neutralized and made up to 50 cc. as described above. This solution will be referred to as fraction No. 2.

The nitroprusside test, iodine titration, and sulphydryl determination were made on the original boiled juices and the two fractions of both the treated and check juice. The results are shown in Table III. It will be noted that fraction No. 2 of the juice of the treated sample reduces iodine, but gives neither the nitroprusside test nor forms hydrogen sulphide from sulphur. It will be noted also that the juice of the untreated tubers shows

TABLE III

THE FRACTIONATION OF THE IODINE-REDUCING SUBSTANCES OF POTATO JUICE INTO A SULPHYDRYL CONTAINING FRACTION AND A NON-SULPHYDRYL CONTAINING FRACTION

	Nitroprusside test			Milli-equiv. of I reduced by 10 cc.			SH content by sulphur reduction. Milli-equiv. SH in 10 cc.		
	Orig. juice	Fract. No. 1	Fract. No. 2	Orig. juice	Fract. No. 1	Fract. No. 2	Orig. juice	Fract. No. 1	Fract. No. 2
Treated with ethylene chlorhydrin	+	++	−	.044	.014	.016	.0035	.0038	.0000
Untreated	−	−	−	.004	.004	.001	.0000	.0000	.0000

an appreciable iodine reduction but no nitroprusside test or reduction of sulphur to hydrogen sulphide, although it should if the iodine reduction were due to a sulphydryl compound. Furthermore, the sulphydryl content of fraction No. 1 of the treated sample as indicated by the formation of hydrogen sulphide, represents only about one-fourth of the total content of reducing substances as shown by the reduction of iodine. It is evident from these results that only a portion of the iodine-reducing substances of the potato are sulphydryl compounds.

SUMMARY

1. The production of hydrogen sulphide from sulphur by the action of cysteine, glutathione, and tissue extracts has been investigated.

2. Theoretical yields of hydrogen sulphide were obtained with solutions of glutathione and yields of 65 per cent with cysteine hydrochloride.

3. When known amounts of glutathione were added to tissue extracts, nearly theoretical recovery of hydrogen sulphide was obtained in the case of yeast, but not in the case of the other extracts used.

4. The reducing power of the tissue extracts as measured by iodine titration was greater than that measured by hydrogen sulphide evolution in all cases except with yeast. This was true even when the hydrogen sulphide values were corrected for the recovery obtained by adding glutathione to tissue extracts.

5. The higher values given by iodine titration are explained by the presence of non-sulphydryl iodine-reducing substances.

6. This explanation is supported by an experiment in which the iodine-reducing substances of the potato were fractionated by lead acetate precipitation into a sulphydryl containing fraction and a non-sylphydryl fraction.

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SOME FACTORS AFFECTING THE EFFICIENCY OF CONTACT INSECTICIDES. II. CHEMICAL AND TOXICOLOGICAL STUDIES OF PYRETHRUM[1]

Albert Hartzell and Frank Wilcoxon

In a series of papers published in 1924–27 Staudinger and co-workers (16, 17) showed that the active constituents of pyrethrum flowers consist of two ketone esters which were called by them pyrethrin I and II. Since then a number of publications have appeared dealing with the toxicity of these materials to insects, their stability, methods of evaluating pyrethrum flowers and preparations biologically, and chemical methods for the determination of the active constituents in the flowers. Although several methods have been suggested for the analysis of the flowers, authorities are not yet in agreement as to the value of these methods for indicating the actual toxicity of samples toward insects.

TOXICITY AND PYRETHRIN CONTENT

The chemical methods that have been proposed may be divided into two groups. In the first group the methods depend on a titration of the acidic constituent of the pyrethrins, with standard alkali (19). In the second group the reducing properties of the pyrethrins are made use of toward sugar reagents, such as alkaline copper solutions (5) or potassium ferricyanide (10). The methods of the first group offer the possibility of determining pyrethrin I and II separately, while in the second group only the total pyrethrin content is estimated. It has been stated that the pyrethrins undergo decomposition on long storage, or on exposure to air and moisture, or to alkali, or in the presence of alcohol, with consequent loss of toxicity (6). It is important, therefore, that a chemical method of analysis should not only indicate the relative toxicity of fresh samples but should correctly show any loss of toxicity which might occur if deterioration should take place.

In 1929 Tattersfield and Hobson (18, p. 435) published a modification of the acid method proposed by Staudinger and Harder (15), which permitted the use of a smaller sample, and compared the results obtained by analysis with those resulting from biological tests on *Aphis rumicis*. A high degree of correlation was shown. However, in 1930 McDonnell *et. al.* (9) stated that the chemical methods compared by them were not all that might be desired. They also performed toxicity tests on aphids, using analyzed samples and found no definite relation between the analytical results and toxicity. This work has recently been reviewed by Glassford (3) who considers that the chemical methods are inadequate as an index of insecticidal power.

[1] Herman Frasch Foundation for Research in Agricultural Chemistry, Paper No. 32.

On the other hand Gnadinger (4) has presented evidence tending to show that the analytical method proposed by him agrees with the method of Tattersfield, and that these methods offer an accurate index of the insecticidal value of pyrethrum flowers. More recently Richardson (12) has found that a determination of pyrethrin I by Tattersfield's short acid method gives results which agree with biological tests on house flies. Pyrethrum which had been artificially deteriorated gave lower analytical re-

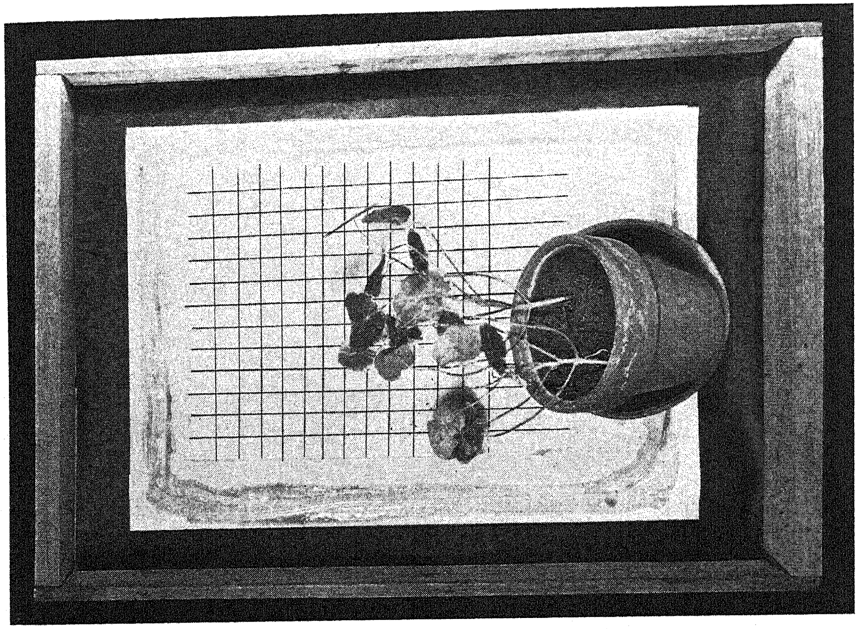

FIGURE 1. Method of placing sprayed nasturtium plants over ruled paper surrounded by tanglefoot border, as used in toxicity tests. View from above of a pot lying on its side in a greenhouse flat.

sults as well as exhibiting a decreased toxicity. In the present paper further evidence is presented showing that Tattersfield's method is reliable as an indicator of the insecticidal value of pyrethrum flowers, and that when the flowers have been deteriorated by various agencies, the chemical analysis will indicate that such deterioration has taken place.

Methods. The analyses were performed as described by Tattersfield, Hobson, and Gimingham (19, p. 278), pyrethrin I and II being determined. N/50 barium hydroxide was used for titration, in order to avoid the effect of CO_2 on titrations with phenolphthalein indicator. The petroleum ether used for extraction of the flowers distilled under 40° C., and the methyl alcohol was purified by distillation over potassium hydroxide. *Aphis*

rumicis L. on dwarf nasturtium plants was the insect used for the biological tests. The sample of flowers (10 g.) was extracted with low boiling petroleum ether in a Soxhlet extractor, and the solvent removed by evaporation under reduced pressure. The residue was dissolved in acetone and made up to 10 cc., and 1.2 cc. of this acetone solution was added to 100 cc.of distilled water. The emulsion formed in this way was sprayed on the nasturtium plants infested with 200–300 aphids, using an atomizer and compressed air at a pressure of 38 cm. of mercury. During the spraying the plants were slowly rotated by hand. Two plants were used for each test. No spreader was used since the presence of an effective spreader such as soap complicates the interpretation of the results owing to its own toxicity. Immediately after the application the plants were placed on flats containing paper surrounded by a tanglefoot barrier and ruled in squares to facilitate counting as illustrated in Figure 1. Counts were made after 24 hours under a binocular microscope. Every aphid on the leaves, stems, and paper was examined and probed with a needle when necessary to decide whether it was alive or dead. Any individual showing movement of antennae or legs was considered alive. Those insects found on the tanglefoot border were considered to be alive. The agreement between duplicate runs by this method was fairly good. In 26 such experiments the average difference between duplicates was 6.4 per cent. The method is probably not capable of as great precision as that used by Richardson (11) for the evaluation of kerosene extracts on house flies, but is better adapted to the testing of aqueous emulsions on plant-infesting insects such as aphids.

In order to determine the relation between pyrethrin content of a spray solution prepared as described above and per cent mortality, the following experiment was performed: A petroleum ether extract of pyrethrum flowers was partially purified with methyl alcohol as described by Staudinger. Analysis by Tattersfield's method showed a pyrethrin content of 64 per cent. Weighed portions of this were dissolved in acetone and a series of emulsions prepared containing increasing amounts of pyrethrins. Toxicity texts were performed using the above solutions, all sprayings being made on the same day. The results obtained are shown in Figure 2. It will be noted that when the pyrethrin content is low, a small increase in concentration is more effective in increasing the mortality, than the same increase at a higher concentration. Obviously attempts to evaluate the pyrethrin content of sprays or flowers should not be carried out at such a concentration that a high percentage mortality is obtained, if accurate results are desired.

In order to determine whether the chemical analysis furnishes a satisfactory means of evaluating toxicity, a number of samples of flowers were obtained from two well known importers. Portions of each sample were submitted to various conditions which it was expected would lead to de-

crease in toxicity. In some cases the sample was exposed in a thin layer to air and sunlight, in other cases to the light of a quartz mercury vapor arc. Another treatment consisted of exposing a portion of the sample to heat in an electric oven. In each case the treated and untreated samples were analyzed by Tattersfield's acid method, and toxicity tests were made on the same day, using an average of 200 insects per plant. The mortality in the checks was about 6 per cent. In this way a series of pairs of samples was obtained, one member of each pair differing significantly in toxicity from the other. The results of the analyses and toxicity tests are shown in Table I.

TABLE I

COMPARISON BETWEEN TOXICITY TO APHIS RUMICIS AND PYRETHRIN CONTENT FOR SEVEN PAIRS OF SAMPLES OF PYRETHRUM FLOWERS

Sample	Kill		Per cent pyrethrins		Total pyrethrins		Difference between pairs by acid method
	Per cent	Difference	I	II	Acid method	Gnadinger method	
Control	37		0.38	0.63	1.01	1.03	
Exposed to sunlight 3 days	27	10	0.24	0.55	0.79	0.81	0.22
Control	55		0.32	0.64	0.96	—	
Exposed to u.v. light 3 hours	33	22	0.23	0.55	0.78	—	0.18
Control	55		0.32	0.64	0.96	—	
Exposed to u.v. light 6 hours	33	22	0.23	0.47	0.70	—	0.26
Control	68		0.28	0.39	0.67	—	
Heated 10 hrs. at 100° C.	37	31	0.23	0.35	0.58	—	0.09
Control	73		0.26	0.49	0.75	0.73	
Heated 40 hrs. at 100° C.	52	21	0.18	0.34	0.52	0.59	0.23
Japanese flowers	41		0.36	0.63	0.99	—	
Turkish flowers	10	31	0.04	0.04	0.08	—	0.91
Fresh flowers	53		0.31	0.67	0.98	0.82	
Flowers 4 yrs. old	28	25	0.24	0.43	0.67	0.64	0.31

Inspection of Table I shows that in every case a positive difference in per cent kill is associated with a positive difference in the analytical figures. There is little correspondence, however, between the actual magnitude of the differences in per cent kill and toxicity for each pair. There are several reasons for this. From the curve previously shown, it may be seen that a given difference in mortality represents a varying difference in concentration depending on the portion of the curve under consideration. In addition to this the figures for per cent kill are subject to a considerable error of random sampling. From our experience it appears that whenever two samples show a significant difference in toxicity by the biological test, the analytical results will confirm this fact. The agreement between the method of Gnadinger and that of Tattersfield is quite good.

STABILITY OF PYRETHRUM

The experiments described above have shown that a loss of toxicity may occur when ground pyrethrum flowers are exposed to ultra-violet light, to sunlight, or to heat. It is well known also that pyrethrum flowers deteriorate with age. It seemed advisable to investigate the effect of these factors on concentrated extracts obtained from flowers.

A sample of flowers was extracted with low boiling petroleum ether, and the solvent removed by means of a water pump. The residue was

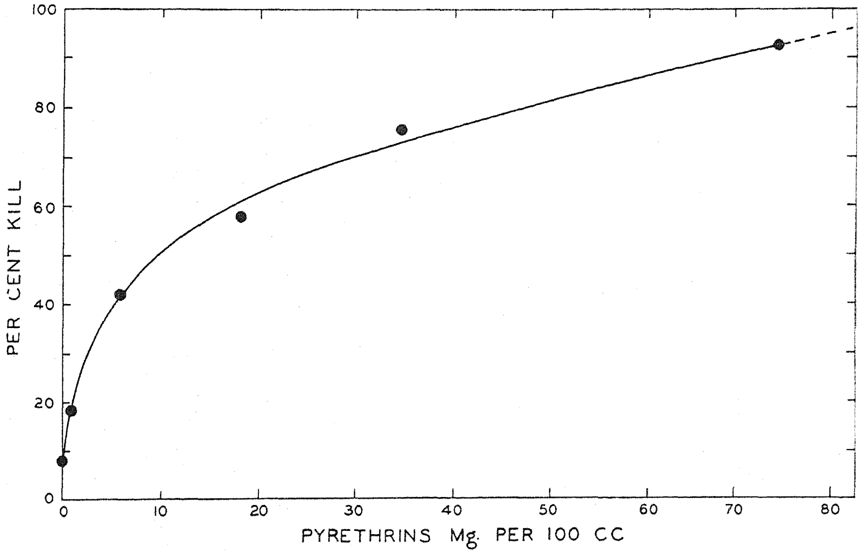

FIGURE 2. Relation between pyrethrin content and percentage kill for pyrethrum solutions applied to *Aphis rumicis* without a wetting agent.

warmed gently with purified methyl alcohol, and the solution, after addition of 10 per cent of water, allowed to stand at $-10°$ C. overnight to precipitate resins. The latter were filtered off and the filtrate evaporated under vacuum. In this way a fairly concentrated extract was obtained which showed 63 per cent total pyrethrins on analysis. A portion of this sample was spread out in a layer 1 mm. thick on a watch glass and placed outdoors in direct sunlight for two successive days. The temperature ranged from 68°–82° F. during the time of exposure. At the end of this period toxicity tests were performed on a weighed portion, the sample being dissolved in acetone and diluted as previously described. A portion of the sample which had not been exposed outdoors served as a control. Duplicate tests on the control gave 53.3 per cent and 55.8 per cent kill, while the exposed sample gave 49.7 per cent and 52.7 per cent kill. The difference between the averages for the control sample and the sample ex-

posed to sunlight was 3.3 per cent. The difference is scarcely significant. A further exposure for 32 hours under similar conditions led to the following results. Controls gave 53.9 per cent and 55.9 per cent kill, and the exposed sample 44.6 per cent and 46.8 per cent kill. The difference between averages is 9.8 per cent kill. Inspection of Figure 2 will show that this difference may involve a loss of nearly one-half of the pyrethrin content. The exposed samples contained much material insoluble in acetone while the control sample did not. Whether this change involves an oxidation or polymerization has not been established.

The fairly rapid deterioration of pyrethrum concentrates, when exposed to air and sunlight, suggested experiments on the permanence of protection afforded by spraying foliage and allowing it to dry previous to infestation with *Aphis rumicis*.

A solution containing 0.2 per cent pyrethrins, far more concentrated than would be used in ordinary practice, was sprayed on nasturtium plants. The plants were allowed to dry for 24 hours in the greenhouse. They were then infested with about 200 aphids and a count of living and dead individuals made after an additional 24 hour period. The kill obtained was 22.7 per cent, which shows that satisfactory protection cannot be obtained in this way, although complete destruction of the pyrethrins requires a considerable period of exposure.

It has been suggested by Harder (6) that alcoholic solutions of the pyrethrins may undergo a loss of toxicity due to a replacement of pyrethlolone in the esters by the alcohol group, and he advises against the use of alcohol solutions of pyrethrum. In order to determine how rapidly such a decomposition might take place, a partially purified extract was refluxed with methyl alcohol for four hours. The solvent was removed in *vacuo* and the toxicity of the residue compared with that of a portion of the original sample, using the method previously described. The sample treated with methyl alcohol gave 61 per cent and 54 per cent kill, while the untreated sample gave 47 and 43 per cent. The difference, while apparently significant, is in the wrong direction to indicate a loss of toxicity.

PHYSIOLOGICAL ACTION OF PYRETHRUM EXTRACTS

Juillet (7) has reviewed the literature to 1924 on the action of pyrethrum on insects, and concludes that pyrethrum acts as a neuro-muscular poison and paralytic agent. He states that penetration of the toxin by way of the mouth is more effective than by application to the integument. More recently Saling (13) has insisted that the active principle of pyrethrum is not a respiratory or a blood poison but a nerve poison causing symptoms that are characteristic of narcotics. Krüger (8) recently reported morphological changes in the hypodermis, muscles, and nerves of Corethra larvae that had been treated with suspensions of pyrethrum

flowers. The ventral nerve ganglia of treated larvae, he states, showed vacuoles which were not present in the untreated larvae.

As no chemical method is known for detecting the presence of pyrethrins in the body of an insect, the work on the physiological response in the present paper was confined to observations of the symptoms produced by pyrethrum extracts principally on the rose chafer (*Macrodactylus subspinosus* Fabr.), the tomato worm (*Phlegethontius quinquemaculata* Haworth), and the cockroach (*Periplaneta americana* L.).

Many of the previous observations on the toxicity of pyrethrum to cold blooded animals have involved the use of pyrethrum flowers. It appeared of interest to investigate the action of the concentrated products described in this paper on a variety of cold blooded animals to determine if any could be found that were not affected. Juillet (7, p. 120) states, for example, that the meal worm (*Tenebrio molitor*) survived 24 hours submergence in pyrethrum flowers. The animals tested with our extracts included representative species of ten orders of insects, spiders, centipedes, millipedes, sow bugs, snails, earthworms, and frogs. These extracts when applied to the integument caused death in all cases including *Tenebrio molitor* L.

By confining rose chafer adults in such a way that a rear leg could not be brought in contact with the rest of the body and by placing a drop of concentrated pyrethrum extract on a tarsus, it was possible to demonstrate that pyrethrum intoxication and death could be brought about without the liquid coming in contact with the head, thorax, spiracles, or other vulnerable parts of the body. In a similar way if a drop of pyrethrum extract is placed in the center of the pronotum of a cockroach, care being taken that none of the liquid reaches the ventral surface of the body, the individual in a short time begins moving its mandibles and cleans its antennae as though some irritating substance was present. Apparently the sense organs are excited although the material is not in actual contact with the antennae or mouth parts.

These observations suggested the advisability of making a study of the stimulating effect of pyrethrum extracts when placed on various regions of the body. For this purpose larvae of the common tomato worm were selected because of their large size and the fact that the segments are plainly visible. Larvae of approximately the same age and size were used in these tests. The time of vomiting was taken as an index of intoxication. In all tests ten individuals of approximately the same age and size were used and the experiments were repeated at least three times. Unless otherwise indicated the experiments were conducted at room temperatures. A total of 175 larvae were used.

It was found that when the drop of extract was placed on the head of a caterpillar that intoxication took place in an average time of eight min-

utes, but when the drop was placed on the dorsum of the last abdominal segment intoxication occurred in an average time of 25 minutes. When the extract was placed on segments intermediate between the head and the last abdominal segment in a series of larvae the time required for intoxication was intermediate. This suggests that there is an axial gradient in toxicity to pyrethrum as one passes from the head region to the caudal region of the body. This is in accord with Child's (2) observations for lower animals. Buchmann (1) by means of subcutaneous injections has demonstrated a like phenomenon with *Periplaneta americana*.

When a drop of extract is placed on the dorsum of the last abdominal segment of a full-grown larva the individual crawls in a normal manner for a period of about 30 minutes, following which the last pair of prolegs are affected, the larva lifting the last ventral segment without gripping with the prolegs. Five minutes later the last abdominal segment becomes paralyzed and the larva rolls over when attempting to turn around, but recovers its normal position in crawling. A minute later the larva vomits, rolls over, and is unable to crawl. It continues to vomit and crawl for a period of 15 minutes, raising the head and fore part of the body in the typical defense action. The movements now become very uncoordinated and violent for a period of 30 minutes after which they become much less violent. The larva now is unable to crawl and continues to lie on its side and make feeble attempts to regain the crawling position. Death usually occurs in 24 hours from the time that the extract is applied. The action of pyrethrum extract is much more rapid on partly grown caterpillars. Intoxication may take place in five minutes with small larvae while with full grown larvae it usually required about 30 minutes.

When a dose of 1/50 cc. of pyrethrum extract was injected into the last abdominal segment of a larva, typical pyrethrum intoxication occurred in two minutes. This indicates that pyrethrum extract is a violent poison when injected into the body cavity and that the symptoms appear in a very short time as compared with external applications of the extract. The delay in the appearance or symptoms may be due to a time factor involved in reaching the higher nerve centers or it may be due to the length of time required for a sufficient amount of the poison to penetrate the integument of the insect and enter the blood stream.

Both ethyl thiocyanate and methyl isothiocyanate when placed on the last abdominal segment of a tomato worm larva caused symptoms not unlike pyrethrum intoxication and resulted in death in 14 hours. The interesting observation was made that placing a drop of C. P. nicotine on a similar position on a larva did not lead to the death of the individual although violent intoxication took place. The fact that certain organic compounds not related to the pyrethrins produce symptoms of intoxication similar to that caused by pyrethrum, suggests the possibility that a search

for simpler compounds producing similar action may be a fruitful field for future investigation.

Relation of temperature to toxicity. It was found that if the body temperature of a treated tomato worm larva was lowered considerably (5° – 10° C.) below that of room temperature that symptoms of pyrethrum intoxication were retarded. When treated larvae were placed in an oven at 52° C. for a period of ten minutes respiratory movements were greatly accelerated beyond those of the check which had received no pyrethrum extract.

The relation of temperature to the action of pyrethrum on insects was clearly shown in the case of the rose chafer. Three series of ten adults were sprayed with 0.005 per cent pyrethrum extract. An hour and a half later when all were moribund one lot was placed in direct sunlight at a temperature of 42° C. The other two lots of beetles were placed in shade at a temperature of 31° C. The adults that were placed in the sun revived in about 30 seconds, moving their legs and attempting to right themselves, and two individuals recovered sufficiently to fly away. After an exposure to sunlight for 80 seconds all movement ceased. The beetles that remained in the shade continued to be moribund. This would indicate that both the forces involved in bringing about death and in bringing about recovery are accelerated by a rise in temperature. If the insects have received a dose insufficient to kill, recovery is more rapid, but if on the other hand the dose is lethal, death occurs more rapidly at the higher temperature. John B. Smith (14) apparently noted a similar effect but the dose applied was sublethal. In order to establish that the effect was thermal and not due to light a similar lot of adults was placed in an oven at 40° C. The results in every respect were the same as those obtained with sunlight.

SUMMARY

1. A method is described for the determination of comparative toxicity to *Aphis rumicis* of samples of pyrethrum flowers without the use of a wetting agent.

2. The average deviation between duplicate determinations made on the same day by this method was 6.4 per cent kill. The relation between pyrethrin content and toxicity has been determined by this method for a series of dilutions of an analyzed pyrethrum concentrate. The relation is non-linear, and the bearing of this on toxicity experiments is pointed out.

3. A loss in pyrethrin content of pyrethrum flowers was caused by heat, sunlight, ultra-violet light, and natural aging. In all cases a decrease in pyrethrin content as determined chemically corresponded to a decreased toxic effect on insects. It appears that whenever two samples show a significant difference in toxicity by the biological test, the analytical results will confirm this fact.

4. Partially purified pyrethrum concentrates, exposed in a thin layer to sunlight, showed a loss in toxicity accompanied by the formation of insoluble material.

5. No lasting protection was afforded by spraying nasturtium plants with pyrethrum solutions, and infesting them with *Aphis rumicis* after the plants had dried.

6. Refluxing a pyrethrum solution in methyl alcohol for four hours did not result in a significant loss in toxicity.

7. Pyrethrum concentrates were toxic to a number of cold blooded animals including species representing ten orders of insects when applied externally to the integument. Such concentrates were toxic even when applied locally to regions of the body far removed from vital organs. These concentrates injected into the body cavity led to almost immediate occurrence of toxic symptoms.

8. Preliminary results indicate that there is an axial gradient in susceptability to pyrethrum when extracts are applied to the tomato worm larvae (*Phelegethontius quinquemaculata*), the anterior region being most susceptable.

9. When rose chafer adults (*Macrodactylus subspinosus*) in a moribund state from pyrethrum intoxication were exposed to a higher temperature, the processes of recovery and death were both accelerated.

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CONDITIONS AFFECTING NITRATE REDUCTION BY PLANTS

Sophia H. Eckerson

In the course of several years' study of the nitrate-reducing ability (reducase activity) of different plants certain facts concerning light and mineral nutrition in relation to reducase activity have become sufficiently clear to make a preliminary report desirable. It is of course necessary to know the reducase activity in the different plants before attempting to study the effect of varied conditions. In the plants so far studied there seems to be a general range which is more or less characteristic for the genus, and there is a variation within this range with the stage in development. For instance, reducase may be generally high as in the tomato, extremely low as in the cranberry, or intermediate as in the Biloxi soybean. The soybean shows the most pronounced variation with development (Table I). For each genus there is also a characteristic distribution of reducase in the organs of the plant. Reducase may be present in tops and roots in about equal amounts as in the tomato or it may be localized chiefly in the tops as in the soybean, or chiefly in the fine roots as in the asparagus and the apple. As there are wide differences in the amount and distribution of reducase under ordinary conditions so there are differences in the degree of the effect on the different plants when the conditions are varied. The direction of the effect, that is either increase or decrease, was the same for all the plants tested.

METHODS

Species used. The following species were used: apple (*Pyrus malus* L. var. Stark); Martha Washington asparagus (*Asparagus officinalis* L. var. *altilis* L.); beet (*Beta vulgaris* L. var. Crosby's Egyptian); Copenhagen Market cabbage (*Brassica oleracea* L. var. *capitata* L.); cranberry (*Vaccinium macrocarpon* Ait. varieties Centennial and Howes); lettuce (*Lactuca sativa* L. var. New York); peach (*Prunus persica* (L.) Stokes var. Greensboro); soybean (*Glycine max* Merr. var. Biloxi); tomato (*Lycopersicon esculentum* Mill. varieties Bonny Best and Marglobe); and wheat (*Triticum aestivum* L. var. Marquis).

Determination of reducase. The method used to determine reducase in plant juices was described fully in a previous paper (2). Briefly it is a measure of the amount of reduction of nitrate to nitrite by a definite amount of plant juice in slightly alkaline solution in the presence of glucose in a given time at a definite temperature, using toluene to prevent bacterial action. At the end of the period the amount of nitrite is determined by means of the sulphanilic acid reagent (2, p. 198) and is recorded in terms of nitrogen as nitrite per cubic centimeter of juice.

Precaution. In case a yellow color develops instead of the clear red given by nitrites with this reagent, it is due to too much nitrite for the amount of the reagent. A new sample is diluted until the red color is obtained with the reagent. The depth of color in the sample is compared with the standard (0.001 mg. N as nitrite) using a colorimeter. If it is thought that some of the red color may be due to anthocyanin in the acid solution, add one or two drops of acetic acid to the sample to acidify before adding the reagent, and note the color, if any appears.

To determine the reducase activity of plants whose tissues are low in moisture, containing therefore but little extractable juice, water extracts are made as previously described for apple tree tissue (3). The reduction of nitrate by the extract is carried out in the same way as reduction by the juice but is recorded as nitrite nitrogen per gram of fresh tissue. The figures are smaller in this case but the course of the variations is the same as when given in terms of one cubic centimeter of juice. When the experimental conditions tend to decrease the moisture in the plant, the percentage decrease in reducase appears to be slightly greater on fresh weight basis than on the juice basis, as is shown in Table III. However, with one exception, the variations are small and no real difference between the two methods is indicated.

RESULTS

EFFECT OF DECREASED LIGHT

The effect of decreased light upon reducase in Biloxi soybean[1] is shown in Tables I to III. For the series given in Table I the seeds were planted in sand in large pots. One lot was exposed to full daylight, another to daylight

TABLE I

EFFECT OF DECREASED LIGHT UPON REDUCASE IN BILOXI SOYBEAN

Date 1930	Mg. N as nitrite per cc. juice in 17 hrs. at 37° C.			
	Long day according to season		Short day by darkening from 4 P.M. to 8 A.M.	
	Tops	Roots	Tops	Roots
March 10	0.0011*	—	0.0011*	—
" 17	0.160	0.0014	0.0037	0.0011
" 24	0.450	0.015	0.003	0.0015
" 31	1.000	0.020	0.040	0.0018
April 7	0.190	0.005	0.080	trace
" 14	0.270	0.002	0.0025	0.0009
" 21	0.010	0.0015	0.0022	0.0013

* Germinating seeds.

[1] The plants of Tables I to IV and VI to VIII were from series grown for studies of nitrogen metabolism at New Jersey Experiment Station and were made available for reducase determinations by Nightingale, Schermerhorn, and Robbins.

for a shorter period by darkening from 4 p.m. to 8 a.m., making an 8-hour day. In the plants exposed to full daylight (columns 2 and 3) the nitrate-reducing ability rose from extremely low in the germinating seed, to a maximum at about the third week, then it decreased to the sixth week. While the general course of rise and fall in the root is similar to that in the top, reducase is always very low in the root.

In the short day plants (columns 4 and 5) reducase was markedly lower. The two-week plants had but little more than the amount originally in the germinating seed. At the fourth week reducase was a little higher, then it decreased to the sixth week. For four of the six weeks the amount was approximately three, one, five, and two per cent of that in the long day plants for the same week, showing a decrease of 95 to 99 per cent. For the two weeks, April 7 and 14, the decrease was 60 and 70 per cent. At the sixth week the plants were only six or eight inches high, with small yellowish leaves and stiff stem. The storage cells of the stem were filled with starch and contained much nitrate, indicating that nitrate was not being assimilated (4).

Another series of the Biloxi soybean was grown under four different light exposures, both with and without nitrate; (a) grown in full daylight (long day light); (b) grown in a frame covered with Fruit of the Loom muslin[2] (long day shade); (c) grown in daylight, but darkened from 4 p.m. to 8 a.m. (short day light); (d) grown in a muslin-covered frame (short day shade). Table II shows the reducase activity in the plants of this series of eight lots at the fifth and sixth week (April 20 and 28). The amount of reducase in the complete nutrient long day light plants (columns 2 and 3) and in the short day light plants (columns 6 and 7) was similar to that found in the plants grown the previous year at about the same stage of development (Table I, April 14 and 21). The decrease in reducase in the short day plants on April 20 was about 97 per cent and on April 28, when reducase had become low in the long day plants, the decrease was 71 per cent.

The amount of reducase in the long day shade plants (columns 4 and 5) and in the short day shade plants (columns 8 and 9) was considerably less than in the corresponding light plants. Specifically, shading with the muslin which transmits only about 34 per cent of the radiant energy de-

[2] Dr. J. M. Arthur measured the transmission of this muslin and has supplied the following data:

Total energy transmitted using a 50 watt frosted incandescent lamp and a pyrheliometer at normal incidence is 34.3 per cent. When measured similarly, except using a filter which removes much of the infra red, the transmission is 30.2 per cent. When a Weston photronic cell is used which is sensitive only to the visible region (maximum sensitivity near wavelength 580 $m\mu$) the transmission is 30 per cent. This difference is due apparently to the selective transmission of the cloth. That is, it transmits more in the infra red than in the visible region. The mesh count when bleached is 86×88 (80×90 on the loom).

creased reducase in the tops of both the long day and the short day plants 64 to 69 per cent. When the amount in the roots was already extremely low, as in the short day plants and at a late stage in the long day plants, shading did not decrease it further.

The percentage decrease in reducase in the tops under the different light exposures on April 20 is given in Table III. Since the decrease in the whole plant did not vary more than two per cent from the decrease in the tops, the figures for the roots were omitted. The decrease of reducase in the long day shade plants was 66 to 68 per cent, while the decrease in the short day light plants was 98 to 99 per cent on either the juice basis or the fresh

TABLE II

EFFECT OF DECREASED LIGHT UPON REDUCASE IN BILOXI SOYBEAN

Date 1931	Mg. N as nitrite per cc. juice in 17 hrs. at 35° C.							
	Complete nutrient							
	Long day according to season				Short day by darkening from 4 P.M. to 8 A.M.			
	Light		Shade		Light		Shade	
	Tops	Roots	Tops	Roots	Tops	Roots	Tops	Roots
April 20	0.160	0.012	0.054	0.004	0.0028	0.003	0.001	0.0033
" 28	0.012	0.0033	0.0037	0.0083	0.0029	0.0015	0.001	0.0067
	Minus nitrate							
April 20	0.20	0.017	0.087	0.003	0.0018	0.0046	0.003	0.0037
" 28	0.028	0.003	0.0027	0.0027	0.025	0.0039	0.0028	0.0033

weight basis. These figures are typical for both series. When the Biloxi soybean was grown under sunlight having the intensity reduced to about one-third by shading, reducase was decreased 64 to 69 per cent but when grown in an 8-hour day and 16-hour night reducase was decreased 95 to 99 per cent.

The amounts of reducase in the minus nitrate and in the complete nutrient plants were similar. Actually in many cases the figures are a little higher for the minus nitrate plants. It is safe to say, therefore, that nitrate deficiency did not decrease reducase in these soybean plants.

The general aspect of the plants was that of the series described by Nightingale, Schermerhorn, and Robbins (7, p. 4, Fig. 1). The short day plants were about six inches high with small yellow green leaves, and stiff stems having their storage cells filled with starch. The plus nitrate plants contained much nitrate. The minus nitrate plants of course had none. The long day plants given complete nutrient were 18 to 20 inches high, with large dark green leaves.

It is not understood why with eight hours of good light still available cutting off the few hours before 8 a.m. and after 4 p.m. should have such a drastic effect on reducase activity. It seems possible that the effect may be due to the increased period of darkness rather than to the shortened light period. It may be that processes are set up during the long night which retard synthesis of reducase or which hasten the breaking down of that already formed, and that these detrimental processes are not overcome during the short day. This is speculative of course yet there is some evidence that reducase decreases more rapidly in plants in continuous darkness than in the light. It is evident that more facts on the effect of darkness are needed.

TABLE III

PERCENTAGE LOSS OF REDUCASE IN BILOXI SOYBEAN UNDER DECREASED LIGHT

Condition	Reducase values		Per cent loss due to decreased light	
	Per cc. juice	Per gram fresh wt.	On juice basis	On fresh wt. basis
	Complete nutrient			
Long day				
Light	0.160	0.070	—	—
Shade	0.054	0.022	66.3	68.6
Short day				
Light	0.0028	0.0006	98.3	99.1
Shade	0.001	0.00013	99.4	99.8
	Minus nitrate			
Long day				
Light	0.200	0.090	—	—
Shade	0.087	0.017	56.5	81.1
Short day				
Light	0.0018	0.00036	99.1	99.6
Shade	0.003	0.0003	98.5	99.7

The effect of decreased light upon reducase in the tomato is shown in Tables IV and V. The plants of the series given in Table IV were transferred to sand when eight inches high and were watered with complete nutrient solution. One lot was exposed to full daylight (long day), another lot was darkened from 4 p.m. to 8 a.m. (short day). At the third week, February 18, reducase was lower in the short day than in the long day plants. Two weeks later, March 3, reducase was much lower in the short day plants. There was none in the top, but still a small amount in the roots (columns 6 to 9).

While reducase in the short day (long night) tomato is greatly decreased it does not become as low as in the Biloxi soybean. In 1922 Nightingale (4) showed that the effect of short day on nitrogen metabolism of the tomato was less than on the soybean.

TABLE IV

EFFECT OF DECREASED LIGHT UPON REDUCASE IN BONNY BEST TOMATO

Date 1930	Mg. N as nitrite per cc. juice in 17 hrs. at 50° C.							
	Long day according to season				Short day by darkening from 4 P.M. to 8 A.M.			
	Leaf		Stem	Root	Leaf		Stem	Root
	Blade	Petiole			Blade	Petiole		
Feb. 18	0.30	0.001	0.021	0.60	0.20	0.0013	0.02	0.051
Mar. 3	1.55	0.002	0.007	0.10	0.00	0.0000	0.00	0.055

The amount of reducase found in series of tomato plants grown in the different seasons through three years is given in Table V. In general reducase is high in both tops and roots from February to October (columns 2 and 3). From November to January it is low as shown in columns 5 and 6. A period of cloudy weather decreases the reducase at any season of the year but the effect is more noticeable during the short days. When most of the days were bright as in December 1929 and November 1930 there was

TABLE V

REDUCASE IN BONNY BEST TOMATO AT THE DIFFERENT SEASONS THROUGHOUT THE YEAR

Mg. N as nitrite per cc. juice in 17 hrs. at 35° C.						
February to October			November to January			
Date	Tops	Roots	Date	Tops	Roots	Remarks
1930			1929			
Feb. 12	0.25	0.56	Nov. 11	0.130	0.150	Sunlight one week; no sun three weeks
" 18	0.40	0.20	" 18	0.005	0.001	
" 24	0.11	0.40	" 26	0.0006	0.0008	
1930			1929–30			
Mar. 24	0.50	0.42	Dec. 12	0.0015	0.0013	
" 25	0.66	0.40	" 18	0.060	0.100	
" 28	0.55	1.11	" 26	0.050	0.037	
1931			" 30	0.095	0.001	
May 11	0.88	0.52	Jan. 9	0.070	0.100	
" 18	0.80	0.25	1930			
" 28	0.11	0.10	Nov. 4	0.060	0.130	
June 3	0.64	0.36	17	0.015	0.065	Sunlight three weeks; no sun one week
" 22	1.25	0.88	" 21	0.016	0.050	
1929			Dec. 9	0.004	0.028	
Aug. 13	0.71	0.91	1931			
" 20	0.85	—	Oct. 26	0.006	0.0016	
" 27	1.00	0.79	Nov. 2	trace	0.010	
Sept. 3	0.75	1.37	" 16	0.0008	0.001	
" 10	0.90	0.57	Dec. 4	0.0000	0.0018	
1930			1932			
Oct. 21	0.66	0.14	Jan. 4	0.008	0.024	Feeble sunlight one day; no sun seven days
" 28	0.09	0.30	" 7	0.000	0.004	
Nov. 2	0.07	0.13	" 9	0.000	0.030	

a fair amount of reducase in either the roots or the tops. When most of the days were dark as in November 1929 and early January 1932, reducase was extremely low.

In the periods of clear weather in November and December the amount of reducase, though low, remained about the same for the four or five weeks of the experiment. This seems to indicate that under these conditions as well as in artificial short day there is in the tomato continuous synthesis of small amounts of reducase replacing what has been used up in the reduction of nitrates.

Reducase decreased during November in other plants in the greenhouse also. Marquis wheat is a striking example. Wheat seedlings grown in flats and harvested when about ten inches high were used in 1929 as a source of the nitrate-reducing substance, in a study of its properties. The amount of reducase in the tops was uniformly high. The juice reduced an amount of nitrate which, in 17 hours at 35° C., yielded 2.00 to 3.00 milligrams of nitrite nitrogen per cubic centimeter. But in late October the reducase activity decreased and it became low (for young wheat) in November. The values obtained were: October 26, 1.30; October 31, 0.80; November 6, 0.14; November 11, 0.16; and December 2, for three flats, 0.16, 0.18, 0.15. Growth of these November and December plants was slow compared with the rapid growth in August and September.

EFFECT OF MINERAL NUTRIENT DEFICIENCY UPON REDUCASE

The effect of mineral nutrient deficiency upon reducase is given in Tables VI to IX. Vigorous young plants were transplanted to sand. One lot was watered with complete nutrient solution, another lot with nutrient solution lacking the element to be studied. The plants of Tables VI to VIII were from the series which were being studied by Nightingale and his co-workers. The reference to their work is given in each case.

The effect of potassium deficiency upon reducase in beet, cabbage, and lettuce is shown in Table VI (7, p. 33). About four weeks after the plants

TABLE VI

EFFECT OF POTASSIUM DEFICIENCY UPON REDUCASE IN BEET, CABBAGE, AND LETTUCE

Plant	Mg. N as nitrite per cc. juice in 17 hrs. at 50° C.				
	Date 1930	Plus potassium		Minus potassium	
		Tops	Roots	Tops	Roots
Beet	Feb. 24	1.20	2.50	1.11	0.53
"	Mar. 10	1.53	0.06	0.008	0.02
"	Mar. 10	—	0.25*	—	—
Cabbage	Feb. 24	1.70	0.50	1.40	0.08
Lettuce	Feb. 24	2.00	2.40	0.71	0.19

* Storage root.

had been shifted to sand (February 24) reducase was lower in all the minus potassium plants than in the plus potassium. The minus potassium lettuce especially had much less ability to reduce nitrate than the plus potassium plants. Two weeks later (March 10) the plus potassium beets had storage roots nearly two inches in diameter, and large green leaves. Reducase was

TABLE VII

EFFECT OF CALCIUM DEFICIENCY UPON REDUCASE IN THE MARGLOBE TOMATO

Date 1930	Mg. N as nitrite per cc. juice in 17 hrs. at 35° C.					
	Plus calcium		Minus calcium		Minus calcium continuous darkness July 1 to 8	
	Tops	Roots	Tops	Roots	Tops	Roots
July 1	1.34	0.20	0.60	0.03	—	—
July 8	0.50	0.66	0.15	0.003	0.008	0.01

high in the tops but had decreased greatly in the roots, especially in the fibrous roots. There was a fair amount in the storage root. The minus potassium plants had small red leaves and a slightly thickened tap root (no storage root developed). The tops as well as the roots had lost most of their reducase.

The effect of calcium deficiency upon reducase in the tomato is given in Table VII. The plants were transplanted to sand June 17 (5). July 1 some of the minus calcium plants were transferred to a dark room and kept in

TABLE VIII

EFFECT OF SULPHATE DEFICIENCY UPON REDUCASE IN THE TOMATO

Date 1931	Mg. N as nitrite per cc. juice in 17 hrs. at 35° C.			
	Plus sulphate		Minus sulphate	
	Tops	Roots	Tops	Roots
June 23	1.42	0.46	0.126	0.259
June 30	1.38	1.88	0.008	0.150
	0.172*	—	0.178*	—

* Small fruits.

continual darkness until July 8. Three weeks after transplanting to sand (July 8) reducase, in the minus calcium plants in the light, had become very low in the roots though it was still fairly high in the tops (columns 4 and 5). Calcium deficiency causes severe injury to the tomato and in this series the stem tips died shortly after July 9 (5, p. 609). The figures in columns 6 and 7 show that the calcium deficient plants after one week in darkness had less reducase than those which had been in the light.

The effect of sulphate deficiency upon reducase in the Marglobe tomato is shown in Table VIII. The plants were transplanted to sand May 12 (8). At the sixth and seventh weeks (June 23 and 30) reducase was lower in the minus sulphate than in the plus sulphate plants. Deficiency of sulphate produced no apparent injury to either tops or roots. The appearance of the plants is shown by Nightingale, Schermerhorn and Robbins (8, Fig. 1).

Bonny Best tomato plants, transplanted to sand May 2, were separated into four lots and watered with nutrient solution: complete nutrient, minus sulphate, minus calcium, and minus potassium. The amount of reducase in the different lots is given in Table IX.

TABLE IX

EFFECT OF MINERAL NUTRIENT DEFICIENCY UPON REDUCASE IN BONNY BEST TOMATO

Date 1931	Mg. N as nitrite per cc. juice in 17 hrs. at 35° C.							
	Complete nutrient		Minus sulphate		Minus calcium		Minus potassium	
	Tops	Roots	Tops	Roots	Tops	Roots	Tops	Roots
May 11	0.88	0.52	—	—	—	—	0.237	0.550
" 18	0.80	0.25	0.050	0.060	0.66	0.08	0.0016	0.011
" 28	0.11	0.10	0.016	0.170	0.038	0.005	0.0035	0.0015
June 3	0.64	0.36	0.285	0.070	0.015	0.002	0.0023	—
" 22	1.25	0.88	0.200	0.008	0.0005	0.0006	0.0023	0.0021

The minus sulphate plants (columns 4 and 5) had less reducase than those given complete nutrient, but there was a fair amount in either tops or roots throughout the seven weeks. The plants were as tall as those given complete nutrient but had a thin stiff stem and smaller leaflets on a long rachis. The storage cells of the lower and middle stem contained much starch and nitrate.

The minus calcium plants (columns 6 and 7) had much less reducase. It decreased first in the roots, then more slowly in the tops. Three weeks after transplanting to sand (May 28) it was already very low in the roots and low in the tops. The next week (June 3) the plants were beginning to show the characteristic injury due to calcium deficiency described by Nightingale and his co-workers (4). Three weeks later (June 22) the stem tips were dead and the roots were in poor condition. Reducase was extremely low as might be expected.

Reducase decreased most rapidly in the minus potassium plants (columns 8 and 9), and it remained extremely low until the experiment was discontinued at the seventh week. In appearance the plants resembled nitrate deficient or phosphate deficient plants except that they were taller. There was much nitrate in the middle stem and more starch than in the complete nutrient plants.

It was shown in a previous paper (2) that the presence of phosphate in the tissues of the tomato is necessary for reducase activity. When phosphate was deficient, reducase decreased rapidly and disappeared before there was any visible injury of the protoplasm. The effect of potassium deficiency on reducase was similar to that of phosphate deficiency. Reducase decreased rapidly, becoming extremely low while the protoplasm was still in good condition. The later injurious effects on the cells are distinct and characteristic. It seems that both phosphate and potassium are necessary for synthesis of reducase. In the calcium deficient plants reducase decreased chiefly after injury to the cell walls and protoplasm due to lack of calcium. It is thought that the effect on reducase synthesis is secondary. In the sulphate deficient plants reducase decreased slowly to 0.15 or 0.20 and continued at about that range for some weeks. Apparently synthesis of reducase was retarded but not inhibited as it appears to be when either phosphate or potassium is lacking. Nitrate deficiency seems to have no effect on reducase (2).

ADDITIONAL NOTES

Nightingale and Schermerhorn concluded from their studies that in the asparagus most of the nitrate assimilation takes place in the fibrous roots. When the tops were growing rapidly they found nitrates only in the fibrous roots. Also when nitrate was supplied to plants lacking it, nitrites and ammonium appeared in the fibrous roots only. A little later asparagine and amino acids appeared in the roots (6, p. 17). In 1930 some of their plants from a similar series were tested for reducase. The plants had been raised from seed, were less than a year old, and were growing vigorously. Determinations made from April 28 to May 19 gave results in terms of nitrite nitrogen per cc. juice in 17 hrs. at 35° C. as follows:

April 28	tops	trace	fine roots	0.08
May 5	tops	0.01	fine roots	0.15
May 12	tops	0.04	fine roots	0.20
May 19	tops	0.03	fine roots	0.16

The new storage roots had very little; May 5, 0.001, May 12, 0.005. The older storage roots never had more than a trace.

The cranberry[3] was tested for reducase weekly from May 13 to June 30, 1930. No reducase was found in either old or young leaves or in new growth of the stem. There were traces in the bark of the old stem and in the fine roots. Occasionally in the bog plants the amount was measurable and was then approximately 0.0005 in the bark and 0.001 in the fine roots. Addoms

[3] Mature plants from the bog and young plants from the cold frame were supplied by Miss White of Whitesbog, New Jersey and experimental sand culture plants were supplied by New Jersey Exp. Sta. in cooperation with the New Jersey Cranberry Station.

and Mounce (1) state that nitrate could not be detected in the cranberry grown in sand and supplied with nitrate.

Reducase in the apple (3) is located chiefly in the fibrous roots during most of the year, while in early spring it is also high in the developing buds. To find whether distribution of reducase in the peach resembles that in the apple weekly tests were made in November 1930. No reducase was found in buds, bark, or fine roots. At that time the amount in the apple fine roots ranged from 0.03 to 0.05 and there was a little in buds and bark. It seems that in distribution of reducase the peach may differ from the apple. Of course tests must be made at other seasons.

SUMMARY

When the Biloxi soybean was grown in a frame covered with Fruit of the Loom muslin which transmits only about 34 per cent of the radiant energy reducase decreased 64 to 69 per cent, in comparison with the amount in unshaded plants.

When the Biloxi soybean was grown in an 8-hour day and 16-hour night the amount of reducase was only 1 to 5 per cent that in the plants grown in full daylight in April thus showing a decrease of 95 to 99 per cent. The reducase was so low that no reduction of nitrate in the plants was apparent.

In the tomato grown in an 8-hour day and 16-hour night reducase was low. Reducase was low also in the plants grown during November and December. In clear weather the amount of reducase was usually about one-tenth that in the March to September plants, while after a period of dark days the amount was much lower, one-hundredth or less. Microscopic examination indicates that the tomato in an 8-hour day and 16-hour night and during November and December continues to reduce nitrate, though at a greatly decreased rate.

Both phosphate and potassium seem to be necessary for reducase synthesis. When either is deficient there is little or no reducase in the plant and nitrate reduction ceases.

When calcium is deficient there is early injury to the plant, the roots being affected first, then the stem tips. Reducase decreases first in the roots, later and more slowly in the tops, apparently following the cell injury.

When sulphate was deficient reducase decreased slowly to a moderately low amount which was maintained for several weeks. Reduction of nitrate in the plant also decreased gradually. There was no apparent disintegration of the protoplasm.

The presence or absence of nitrate or of starch seems to have no effect on reducase activity. Reducase is high in high nitrogen plants containing some nitrate and a little starch and it is also high in low nitrogen plants containing much starch but no nitrate.

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EFFECT OF ETHYLENE CHLORHYDRIN TREATMENTS ON THE CATALASE, PEROXIDASE, pH, AND SULPHYDRYL CONTENT OF GLADIOLUS CORMS[1]

JOHN D. GUTHRIE, F. E. DENNY, AND LAWRENCE P. MILLER

It has been shown that ethylene chlorhydrin treatments hasten the growth of dormant potato tubers (2) and increase the catalase, peroxidase, pH, and reducing properties of the expressed juice (4). Since ethylene chlorhydrin also breaks the dormancy of freshly harvested gladiolus corms (3), it was thought desirable to examine treated and untreated corms to find what changes were produced by the treatments. This paper reports the results of a series of experiments with corms of four varieties of gladiolus (*Gladiolus* sp. varieties Souvenir, Alice Tiplady, Halley, and Remembrance). Catalase, peroxidase, pH, and capacity to reduce iodine in acid solution were determined on the expressed juice. In some cases, catalase and peroxidase were determined on the dried, powdered tissue. Since preliminary experiments indicated that ethylene chlorhydrin treatments of potato tubers and gladiolus corms resulted in an increase of sulphydryl as indicated by the power of the juice to form hydrogen sulphide from elementary sulphur, this reaction was studied with expressed juice and tissue extracts.

METHODS

The outer husks were removed from the corms and vapor treatments made with ethylene chlorhydrin as described in a previous paper (3). In all cases a 40 per cent solution of ethylene chlorhydrin was used and concentrations are expressed in terms of this solution. After treatment the corms were planted. Analyses were made about two weeks after the experiments were started. Sixteen corms were allowed to grow in the greenhouse in order to see what effect the treatments had on growth.

Juice was obtained by grinding through the nut cutter of a food chopper, squeezing through cheesecloth in a hand press and centrifuging at a high speed until the starch and a gelatinous precipitate were brought down. It was found that this procedure was unsatisfactory in the case of catalase, since with different varieties of gladiolus varying amounts of this enzyme were carried down by the gelatinous precipitate. For this reason, uncentrifuged juice was used for catalase determinations. In certain experiments, tissue was dried, stored, and powdered. Catalase and peroxidase were determined on these dry powders.

For catalase, the apparatus and procedure described by Davis (1) were used except that the Dioxygen was neutralized with calcium carbonate in-

[1] Herman Frasch Foundation for Research in Agricultural Chemistry, Paper No. 35.

stead of sodium hydroxide. The quantity of juice or tissue used was the amount necessary to give a satisfactory reading, and was the same for all determinations in the same series.

The procedure for peroxidase has been described in a previous paper (5). The values are in terms of milligrams of indophenol produced in ten minutes. They are calculated to represent the activity of one cc. of juice, or in the case of dried tissue, 0.1 gram of the dried powder.

The reduction of iodine in acid solution was determined on five cc. of juice, to which ten cc. of 10 per cent trichloracetic acid had been added. Starch was used as the indicator. In some cases the titration was made directly with N/100 iodine, in other cases a definite amount of iodine and two cc. of 25 per cent potassium iodide were added and the excess iodine titrated with N/100 $Na_2S_2O_3$ as recommended by Perlzweig and Delrue (10). The latter method gave higher values and a somewhat more satisfactory end point. Differences when present could be shown by either method.

The power of the juice to reduce elementary sulphur to hydrogen sulphide, a reaction characteristic of sulphydryl compounds, was determined by the lead paper method of McCallan and Wilcoxon (9). Five cc. of juice were used. Both fresh and boiled juice were tried for this reaction. Boiled juice is more effective than fresh juice. This is due to the inhibiting effect of oxidase in the fresh juice (6). In later experiments the capacity of tissue extracts to form hydrogen sulphide from sulphur was investigated. The tissue was killed by dropping into boiling water, extracted with alcohol, and the amount of hydrogen sulphide produced by the extract determined by absorbing in zinc acetate solution and converting into methylene blue by adding para-amino-dimethylaniline and ferric chloride. This procedure has been described in detail in a previous paper (8).

RESULTS

Experiments with Variety Souvenir

Corms of the variety Souvenir were treated eight weeks after harvesting with different concentrations of ethylene chlorhydrin vapor for various lengths of time. The corms were planted after treatment and analyses made on the juice 14 days after the start of the experiment. Part of the tissue was dried and stored. At a later date this dried tissue was powdered, ground with water, and peroxidase determined on an aliquot of the suspension. The results are shown in Table I. It will be noted that the treatments produced large increases in catalase, peroxidase, and the power of the juice to reduce sulphur to hydrogen sulphide. The effect of two cc. of chlorhydrin per liter for two days is to increase catalase twelve-fold, the peroxidase eight-fold, and the power of the juice to reduce sulphur

TABLE I

EFFECT OF ETHYLENE CHLORHYDRIN TREATMENT ON DORMANT CORMS OF GLADIOLUS VARIETY SOUVENIR

Treatment	pH	N/100 I cc.	Catalase*	Peroxidase		Reduction of S. mg. H_2S in 3 hr.		Wt. of tops after 11 weeks, grams
				Juice	Dried powder	Fresh juice	Boiled juice	
3 cc. per l. for 3 days	6.03	5.9	30.7	7.75	2.16	.037	.083	28
2 cc. per l. for 2 days	5.97	6.5	38.0	7.05	1.83	.048	.090	31
1 cc. per l. for 1 day	5.93	5.5	3.0	3.19	1.11	.018	.056	144
Check—untreated	5.85	6.7	3.0	0.88	0.77	.000	.026	7

* cc. O_2 in 2 min. by 0.25 cc. of juice.

three-fold. The results on the dried powder show that the peroxidase difference persists even after drying and storing the tissue.

The treatments increase the pH slightly. The treatment of two cc. per liter for two days produced an increase of 0.12. An examination of the data for three other series of treatments on Souvenir shows that two cc. per liter for two days produced increases in pH of 0.21, 0.17, and 0.15. The treatments appear to decrease the iodine titration to a slight extent. An examination of the data from similar series of treatments shows that one cc. per liter for one day probably produces the largest decrease. This treatment in the series reported in Table I produced a decrease of 1.2 cc. The decreases noted for this treatment in three other series are 0.6, 0.8, and 0.5 cc.

The effect of the treatments on growth is shown by the weight of tops after 11 weeks. The best growth response was with the one cc. per liter for one day treatment. The weight of tops produced in this treatment was 144 g. as compared with 7 g. for the untreated lot. In two other comparable series of treatments of Souvenir the best growth was noted with one cc. per liter for one day. It is interesting that little change in catalase could be shown for this treatment. In the series reported in Table I, where analyses were made after 14 days, no catalase change was noted for the one cc. per liter for one day treatment. In another series, sampled after 12 days, this treatment reduced the catalase value 1.3 cc. and in a series sampled after 8 days an increase of 2.4 cc. was observed. The relation of catalase values to the time of sampling will be discussed in a later part of the paper.

In order to see what were the effects of treatment when the corms were no longer dormant, treatments were made after storage for six months and analyses made 15 days after the beginning of the treatments. The results are shown in Table II. Large increases are produced in peroxidase, in catalase, and in the power of the juice to reduce sulphur. The increases in peroxidase and catalase are not quite as large as in dormant material,

TABLE II

EFFECT OF ETHYLENE CHLORHYDRIN TREATMENTS ON NON-DORMANT CORMS OF GLADIOLUS VARIETY SOUVENIR

Treatment	pH	Catalase*	Peroxidase	Reduction of S. Boiled juice mg. H_2S in 3 hr.	Av. height of plants after 6 weeks, cm.
2 cc. per l. for 3 days	5.95	29.4	5.68	c.140	14
$1\frac{1}{3}$ cc. per l. for 2 days	5.93	21.9	3.95	0.185	17
$\frac{2}{3}$ cc. per l. for 1 day	5.95	11.9	3.20	0.121	33
Check—untreated	5.61	11.7	2.19	0.042	43

* cc. O_2 in 1 min. by 0.5 cc. of juice.

probably because the enzyme activities have increased in the untreated corms. The treatment of non-dormant corms did not increase growth, but retarded it. This is shown by the average height of the plants after six weeks.

Experiments with Variety Alice Tiplady

Corms of variety Alice Tiplady were treated with ethylene chlorhydrin five weeks after harvesting and analyses for peroxidase, reduction of iodine, and reduction of sulphur made on the juice after 12 days. Part of the tissue was dried and stored. This dried tissue was powdered and

TABLE III

EFFECT OF ETHYLENE CHLORHYDRIN TREATMENTS ON DORMANT CORMS OF GLADIOLUS VARIETY ALICE TIPLADY

Treatment	N/100 I cc.	Catalase*	Peroxidase		Reduction of S Juice of frozen tissue mg. H_2S in 3 hr.		Wt. of tops after 8 weeks, grams
			Juice of fresh tissue	Juice of frozen tissue	Unboiled	Boiled	
3 cc. per l. for 3 days	2.3	14.5	9.72	16.5	.010	.055	48
2 cc. per l. for 2 days	2.7	10.1	4.18	11.9	.013	.064	181
Check—untreated	3.0	4.7	2.88	7.1	.002	.036	17

* cc. O_2 in 2 min. by 0.5 g. dry powdered tissue.

catalase determined on the powder. Another part of the tissue was frozen and stored at $-10°$ C. for six weeks. Peroxidase and reduction of sulphur were determined on the juice of this frozen tissue. The results of these analyses are shown in Table III. Peroxidase, catalase, and reduction of sulphur to hydrogen sulphide show large increases. A small lowering is noted in the iodine titration. The effect of this series of treatments on growth is shown by the weight of tops produced after eight weeks. The two cc. per liter for two days treatment brought about the best growth, re-

TABLE IV

EFFECT OF ETHYLENE CHLORHYDRIN TREATMENTS OF PARTIALLY DORMANT CORMS OF GLADIOLUS VARIETY ALICE TIPLADY

Treatment	pH	N/100 I cc.	Catalase*	Peroxidase	Reduction of S. Boiled juice mg. H_2S in 3 hr.	Wt. of tops after 5 weeks, grams
2 cc. per l. for 2 days	6.05	4.6	18.8	3.86	.044	15
1 cc. per l. for 1 day	6.03	4.8	12.7	4.14	.044	44
$\frac{1}{4}$ cc. per l. for 1 day	6.03	5.2	10.3	3.10	.048	53
Check—untreated	5.93	5.7	6.2	2.60	.036	12

* cc. O_2 in 2 min. by 1 cc. of juice.

sulting in a production of 181 g. of tops compared with 17 g. for the untreated lot.

In another experiment, corms were treated ten weeks after harvesting with various concentrations of ethylene chlorhydrin. The results are summarized in Table IV. It will be noted from the weight of tops produced after five weeks' growth that the corms were not very dormant. However, two of the treatments produced an increase in growth. Nine days after the beginning of the experiment analyses were made on the juice obtained from various lots. A large increase in catalase is produced by the treatments. The change in peroxidase is distinct, but not unusually large. A small increase is produced in the power of the juice to reduce sulphur to hydrogen sulphide. An examination of the data from this and other experi-

TABLE V

EFFECT OF ETHYLENE CHLORHYDRIN TREATMENTS ON NON-DORMANT CORMS OF GLADIOLUS VARIETY ALICE TIPLADY

Treatment	pH	Catalase*	Peroxidase	Reduction of S Boiled juice mg. H_2S in 3 hr.	Av. height of plants after 6 weeks, cm.
$1\frac{1}{3}$ cc. per l. for 2 days	5.80	23.0	6.18	0.119	17
$\frac{2}{3}$ cc. per l. for 1 day	5.92	14.0	4.43	0.075	28
Check—untreated	5.71	8.7	3.15	0.045	36

* cc. O_2 in 2 min. by 1 cc. juice.

ments with the same variety shows that the increase in sulphur-reducing power of the juice, although probably significant, is smaller for Alice Tiplady than for other varieties investigated. The peroxidase and catalase changes are also smaller. An increase of 0.12 of a pH unit is produced by the treatment of two cc. per liter for two days. An examination of the data for three other series of experiments not reported here shows that this treatment produced increases in pH of 0.05, 0.07, and 0.06. The two cc. per liter for two days treatment decreased the iodine titration 1.1 cc. An examination of the data for other series in regard to the lowering of the

iodine titrations reveals decreases produced by the two cc. for two days treatment of 0.1, 0.7, 0.4, and 0.4 cc.

In order to learn whether ethylene chlorhydrin produced changes in non-dormant material, corms of Alice Tiplady that had been stored for six months were treated and analyses made after 14 days. The results are shown in Table V. The treatments produced large increases in catalase, in peroxidase, and in the power of the juice to reduce sulphur. A small increase in pH was also produced. As shown by the average height of the plants after six weeks, the treatments of these non-dormant corms retarded their growth.

Experiments with Variety Halley

Corms of variety Halley were treated, nine weeks after harvesting, with ethylene chlorhydrin and analyses for pH, catalase, peroxidase, and reduction of sulphur made on the juice after nine days. The results are shown in Table VI. A large increase in catalase, peroxidase, and power to reduce sulphur was noted. The data also show an increase in pH, but its reality is doubtful on account of the extremely low value for the check indicating that this value may be in error. Repetition of the experiment on another lot of corms gave an increase in pH for the two cc. per liter for two days treatment of only 0.08 of a pH, with a pH of 5.80 for the check. Large increases in catalase and peroxidase were noted in analyses made on two other series of treatments. The effect of the treatments on growth is shown by the weight of tops produced after six weeks. It is evident from the large amount of growth in the untreated lot and the rather small changes produced by the most favorable treatment that the corms were almost out of their dormant period. Treatment of strictly non-dormant corms showed large catalase and peroxidase increases.

TABLE VI

EFFECT OF ETHYLENE CHLORHYDRIN TREATMENTS ON PARTIALLY DORMANT CORMS OF GLADIOLUS VARIETY HALLEY

Treatment	pH	Catalase*	Peroxidase	Reduction of S Boiled juice mg. H_2S in 3 hr.	Wt. of tops after 6 weeks, grams
3 cc. per l. for 3 days	5.98	25.5	33.5	.014	12
2 cc. per l. for 2 days	5.98	29.2	23.3	.017	49
1 cc. per l. for 1 day	5.90	15.2	19.0	.008	66
Check—untreated	5.56	8.7	9.0	.000	36

* cc. O_2 in 2 min. by 1 cc. juice.

Experiments with Variety Remembrance

Corms of variety Remembrance were treated ten weeks after harvesting. At this time the corms were no longer very dormant as shown by the

growth of the untreated lot. The results of analyses made 11 days after the beginning of the treatments are shown in Table VII. Large increases are produced in catalase, peroxidase, and in the power of the juice to reduce sulphur to hydrogen sulphide. Contrary to the results with Souvenir and Alice Tiplady, the iodine titration showed a distinct increase. A possible reason for this is the rather large change in pH, much larger than those observed with Souvenir or Alice Tiplady. This would be in agreement with results of experiments with potatoes, where increase in iodine

TABLE VII

EFFECT OF ETHYLENE CHLORHYDRIN TREATMENTS ON PARTIALLY DORMANT CORMS OF GLADIOLUS VARIETY REMEMBRANCE

Treatment	pH	N/100 I cc.	Catalase*	Peroxidase	Reduction of S. Fresh juice mg. H_2S in 3 hr.	Wt. of tops after 5 weeks, grams
2 cc. for 2 days	6.19	5.0	15.2	7.50	.027	58
1 cc. for 1 day	5.90	4.6	8.4	3.70	.023	54
¼ cc. for 1 day	5.83	4.5	7.5	3.50	.023	33
Check—untreated	5.71	3.4	4.9	2.69	.008	29

* cc. O_2 in 2 min. by 1 cc. juice.

titrations was found correlated with increase in pH (7). Repetition of the same experiment with another lot of corms gave the same relatively large increase in pH of the juice and an increase in the iodine titration.

Catalase at Intervals After Treatment

In the previous experiments, determinations were made about two weeks after treatment. In order to determine whether changes were detectable at an earlier date, catalase determinations were made on treated and untreated corms at shorter intervals after treatment. The tissue was ground with 5 per cent by weight of calcium carbonate in a food grinder, the juice squeezed out with a hand press, diluted to one-tenth of its volume, and 10 cc. taken for catalase determinations. The results are shown in Table VIII. It will be noted that large catalase differences between treated and check lots were found on the fifth or sixth day after treatment. In one experiment with variety Alice Tiplady, in which earlier samples were taken, significant differences were not noted until the fifth day. It will be seen that there is a tendency for the catalase differences to be less in samples taken about the fifteenth day, than in samples taken earlier. Perhaps the catalase differences found in some of the earlier experiments would have been even greater, had the samples for catalase been taken sooner.

TABLE VIII

RESULTS OF CATALASE DETERMINATIONS MADE ON THE EXPRESSED JUICE OF GLADIOLUS CORMS AT INTERVALS AFTER TREATMENT WITH ETHYLENE CHLORHYDRIN

Variety	Treatment	Days after beginning of treatment	Catalase cc. O_2 in 1 min. Treated	Untreated
Alice Tiplady	3 cc. per liter for 3 days	6 10 15	10.8 9.5 6.0	3.7 3.7 4.0
	2 cc. per liter for 2 days	5 9 13	11.1 4.3 3.5	3.5 2.8 2.6
	2 cc. per liter for 2 days	2 3 4 6	2.7 3.8 5.4 9.8	3.7 5.7 4.4 3.5
Souvenir	3 cc. per liter for 3 days	5 11 15	14.5 28.3 12.0	8.0 4.2 5.4
	2 cc. per liter for 2 days	6 9 15	18.7 13.9 5.7	3.8 3.8 4.1
Halley	3 cc. per liter for 3 days	6 11 16	14.4 20.6 11.2	4.1 3.1 2.3
	2 cc. per liter for 2 days	6 10 14	7.9 8.3 6.1	2.6 2.0 1.4

Effect of Treatment on Sulphydryl Content of Tissue Extracts

The most serious objection to estimations of sulphydryl compounds made on the expressed juice of tissues is the possibility of oxidation of these compounds during grinding of the tissue and expressing the juice. This source of error is avoided by dropping the tissue into boiling water prior to extraction. This destroys all enzymes and obviates any enzymic changes in extraction. This procedure has been applied to corms of each variety, treated with various concentrations of ethylene chlorhydrin for different lengths of time. Two weeks after treatment, sulphydryl was estimated by the sulphur reduction method (8). Iodine titrations were also made on the extracts according to the procedure of Perlzweig and Delrue (10). The effect of the treatments on growth of the corms was followed by determining the time required for half of the corms to show sprouts above ground, and by weighing the sprouts produced after a given time. The results are shown in Table IX. Although the amount of iodine reduced by the tissue extracts is not greatly affected by the chlorhydrin treatments, the reduction of sulphur is greatly increased by the treat-

TABLE IX

EFFECT OF ETHYLENE CHLORHYDRIN TREATMENT OF DORMANT GLADIOLUS CORMS ON GROWTH AND ON THE IODINE TITRATION AND SULPHYDRYL CONTENT OF EXTRACTS OF THE TISSUE

Treatment		Variety Souvenir				Variety Alice Tiplady			
cc. per liter	Days exposed	Milli-equiv. I reduced	Milli-equiv. SH by S reduction	Wt. of sprouts after 12 weeks, g.	Weeks for 50 per cent above ground	Milli-equiv. I reduced	Milli-equiv. SH by S reduction	Wt. of sprouts after 10 weeks, g.	Weeks for 50 per cent above ground
3	3	0.065	0.0141	20	10	0.052	0.0079	166	5
2	2	0.065	0.0121	20	11	0.054	0.0071	84	6
1	1	0.058	0.0074	54	10	0.055	0.0044	102	6
0	0	0.064	0.0028	3	14	0.063	0.0027	1	10

TABLE IX (*Continued*)

Treatment		Variety Halley				Varety Remembrance*	
cc. per liter	Days exposed	Milli-equiv. I reduced	Milli-equiv. SH by S reduction	Wt. of sprouts after 7 weeks, g.	Weeks for 50 per cent above ground	Milli-equiv. I reduced	Milli-equiv. SH by S reduction
3	3	0.093	0.0097	56	4	0.101	0.0201
2	2	0.097	0.0104	40	5	0.103	0.0142
1	1	0.098	0.0067	15	7	0.091	0.0072
0	0	0.102	0.0021	0	>13	0.080	0.0930

* Some of the untreated lot and all treated lots rotted before sprouting. Therefore growth data could not be obtained.

ments. These results furnish an excellent demonstration of the superiority of the sulphur reduction method for estimating sulphydryl over the iodine reduction method. Had the iodine reduction method been relied upon, the increase in sulphydryl brought about by ethylene chlorhydrin treatment of the corms would have been missed entirely, and far too high values would have been assigned to the sulphydryl content of gladiolus corms.

SUMMARY

1. Treatment of corms of gladiolus with ethylene chlorhydrin brought about large increases in the catalase and peroxidase content of the expressed juice and of the dried, powdered tissue.

2. The magnitude of the increase in pH of the juice induced by the treatments depended on the variety. The smallest increase was noted with variety Alice Tiplady and the largest with variety Remembrance.

3. Very little change in the power of the juice to reduce iodine in acid solution was noted, except with variety Remembrance. With this variety the treatments increased the iodine-reducing power.

4. The treatments produced large increases in the sulphydryl content of the expressed juice and of extracts of the tissue as measured by the sulphur reduction method.

5. Ethylene chlorhydrin treatments produced increases in peroxidase, catalase, pH, and sulphydryl with both dormant and non-dormant corms.

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EFFECT OF STORAGE ON THE VITALITY OF DELPHINIUM SEEDS

Lela V. Barton

INTRODUCTION

Certain seeds are known to lose their vitality soon after harvest. If such seeds are economically important and especially if there are periodical failures in seed production, it becomes important to find effective methods of storage which will make possible the maintenance of vitality.

This problem has been the subject of scientific investigation for many years. Reports of these investigations are numerous and deal with many phases of the subject. Only those experimental results which deal directly with the effects of various temperatures and sealing upon stored seeds will be discussed here.

In 1897, Cieslar (2) reported that sealed storage of *Picea excelsa*, *Pinus austriaca*, and *P. sylvestris* lengthened the life of the seeds so that those stored in this way showed higher germination percentages than similar seeds stored in open air. In six-year-old *Picea excelsa* he reported a difference in germination of 33 per cent. He also found higher germination energy in *Picea excelsa* after sealed storage.

Haack (7) working on pine seeds confirmed the results of Cieslar. He stated that sealed in comparison with open storage for three years reduces the fall in germinative power 16 to 68 per cent in different samples. He said that this difference was greater in lots of seeds which were inclined to a rapid decrease in germinative power. Haack also tried various storage temperatures and found the lowest one (ice cellar) the best.

According to Heinrich (9), the viability of seeds may be greatly prolonged even in a high temperature (30° C.) when they are artificially dried and stored with air excluded. A low temperature (under 5° C.) without exception favored the viability of seeds stored for a long period even though they had much hygroscopic moisture.

Delavan (4) found a constant cold temperature even with moist atmosphere better than a warm, drier condition for storage of hickory and oak seeds.

Seeds of *Tussilago farfara* which ordinarily lose their vitality in three months were stored by Dorph-Petersen (5). He found that seeds stored above 0° C. lost their germination capacity after four months while samples stored at −15° C. kept unaltered for more than two years.

Joseph (10) reported that the optimum storage condition for parsnip is reached when the seeds are artificially dried and stored air-tight at low temperatures.

Reports of five different investigators in 1931 have furnished additional

data on the problem of storage in relation to temperature and sealing. Spencer (11) found that soybeans and peanuts kept better in cold storage (55° to 60° F.) while Beattie (1) reported that temperatures of 32°, 40°, or 70° F. were about equally effective for storage of peanut seed. Darragh (3) reported experiments with sugar cane seed which showed that packing with $CaCl_2$ and storing in an air-tight receptacle kept the seed viable for as long as 12 months, whereas they ordinarily lost their germination ability in a few weeks. He also found that the lowering of the storage temperature was beneficial for retention of vitality in cane seed. Longleaf pine seeds germinated well a year or even two years after collection when they were stored in sealed glass jars at temperatures of 25° to 35° F. (12). Finally Wilde (13, p. 74) stated that "delphinium seeds lose their vitality very quickly unless they are stored at a uniform cold temperature and even under this condition they often fail to give good germination at the end of the second or third year."

Many seedsmen and gardeners face the problem of how to store delphinium seed to keep it viable for a year or longer. In an effort to aid in the solution of this problem the experiments reported below were performed.

MATERIAL AND METHODS

All seeds were obtained from Vaughan's Seed Company. The main lot was received in December 1926. The following varieties of the genus *Delphinium* were used:

1942B—Annual delphinium. Double. Dwarf. Rocket mixed. Holland grown. 1924 crop.

F1940A—Annual delphinium. Giant hyacinth flowered. Mixed. Holland grown. 1925 crop.

1970A—Annual delphinium. Stock flowered mixed. U. S. A. grown. 1926 crop.

F2002A—Perennial delphinium. Gold medal hybrids. U. S. A. grown. 1924 crop.

2002A—Perennial delphinium. Hybrid, single, mixed. Holland grown. 1925 crop.

2002B—Perennial delphinium. Hybrid, single, mixed. Holland grown. 1926 crop.

Germination tests were made immediately and storage experiments were begun. These tests were made on filter paper in petri dishes at temperatures of 15° C., 20° C., and ice box (approximately 8° C.). As germination occurred, the seedlings were counted and discarded.

On Dec. 12, 1926, samples of each of the lots of annual and perennial seeds were stored as follows:

Room temperature—open and sealed

8° C.—open and sealed

$-15°$ C.—open and sealed

$-15°$ C.—open and sealed after having been dried over CaO for 10 days.

These temperatures were not constant but varied $\pm 4°$ or $5°$ C. A tight stopper covered with paraffin served to seal the seeds in small glass vials. The sealed vials were then placed in large bottles fitted with ground glass stoppers. The open bottles were covered with cheesecloth.

From these containers samples were taken for germination tests at intervals of one to seven months. For these tests a temperature of 15° C. was always used. In some cases additional temperatures were tried. For the most part, tests were made on filter paper.

In order to determine whether the seeds still possessed energy enough to come up through the soil, samples were planted in a greenhouse flat after 39 and 62 months of storage. For each of these plantings new seeds were obtained from Vaughan's Seed Company in order to have controls. These new seeds were harvested in the fall of 1929 and 1931 and the germination tests were made in March and February after harvest.

RESULTS AND DISCUSSION

Temperature Tests

Since delphinium seeds have very rough coats, they mold quite badly when put in germinating conditions. To check this mold they were sterilized with 0.25 per cent uspulun for 30 minutes before they were put in the petri dishes. In spite of this precaution mold appeared at an early date. Parallel tests were run with seeds which had not been treated with uspulun and although the mold appeared earlier, the germination percentage was practically the same as that of sterilized seeds. Hence sterilization was discontinued after 16 months of storage.

In the original germination test three different temperatures were used: ice box (approximately 8° C.), 15° C., and 20° C. Results indicated that the two former were about equally good for germination. Fifteen degrees C. was the only temperature used in subsequent tests up to 16 months of storage when tests were made at ice box temperature, 15° C., 20° C., and 25° C. as well as daily alternating temperatures[1] of 10° and 20° C. and 15° and 30° C. Again ice box temperature and 15° C. proved favorable but there was also good germination at the alternating temperature of 10° and 20° C. (see Table I). In some cases the latter seemed even better than the former. This pointed to the possibility that the alternating temperatures might be better than a constant temperature for the germination of old seeds. Consequently 15° C. and 10° and 20° C. alternation were used for germination tests after the seeds had been stored for 35 and 39 months.

[1] Put at higher temperature eight hours and at low temperature 16 hours each day.

TABLE I

EFFECT OF DIFFERENT TEMPERATURES ON GERMINATION OF DELPHINIUM SEEDS

Seed		Per cent germination									
		Before controlled storage			Stored 16 months at 8° C. (sealed)						
Annual or perennial	Crop	8° C.	15° C.	20° C.	8 °C.	10° C.	15° C.	20° C.	25° C.	10–20° C.*	15–30° C.*
Annual	1924	31	27	1	9	—	10	—	—	17	—
	1925	64	57	19	46	—	43	—	—	61	—
	1926	74	72	10	40	0	68	1	0	72	23
Perennial	1924	0	2	16	0	—	0	—	—	0	—
	1925	7	19	22	1	—	11	—	—	14	—
	1926	41	43	48	30	0	47	35	38	49	37

* Daily alternating temperatures.

Results here indicated that, although slightly better germination was obtained at the alternating temperatures in a number of cases, 15° C. remained a satisfactory temperature for testing the viability of the seeds. Harrington (8) reported that alternating temperatures were less effective than 15° C. for germination of delphinium seeds but the temperatures which he tried were higher than the ones reported here. A reference to Table I will show the effects of different germination temperatures on fresh seeds as well as those which have been stored in different conditions for a period of 16 months.

Storage of Annual Seeds

At room temperature it is very important that the seed containers be sealed if the germinative power of the seed is to be retained. Especially is this true after the seeds have been in storage for 20 months or more. A reference to Figure 1 will make this clear. It should be kept in mind that the 1924 crop of seeds were two years old at the beginning of this experiment and had presumably been stored open at room temperature up to the time the present experiments were begun. Similarly the seeds of the 1925 crop were one year old while those of the 1926 crop were freshly harvested. The expected difference in viability of the seeds as shown by the original as well as later germination tests is clearly shown in Figure 1.

Again it will be seen that the general effect of open and sealed storage at room temperature is the same for all seeds regardless of the age (Table II and Fig. 1). After 20 months of open storage, there is a decline in germinative power until at

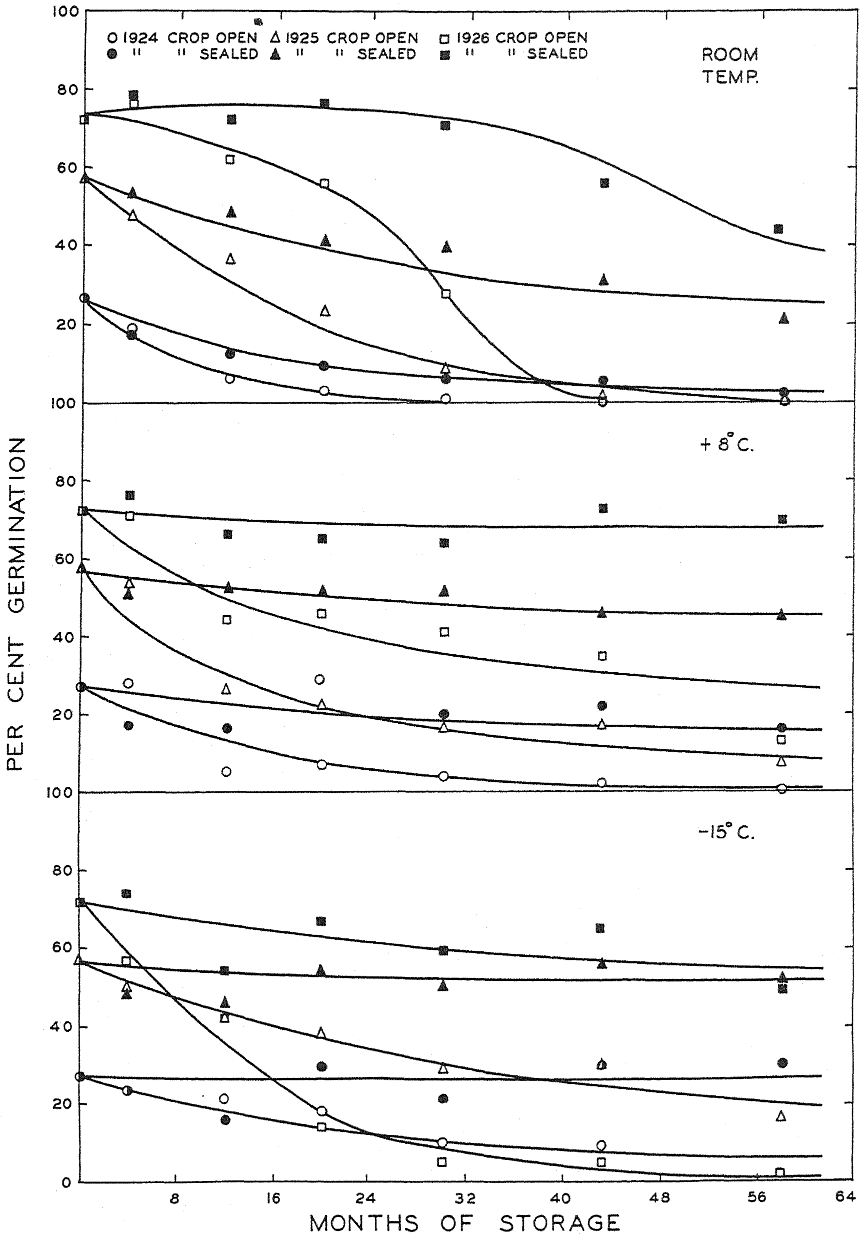

FIGURE 1. Germination curves of annual delphinium seeds after open and sealed storage at room temperature, 8° C., and $-15°$ C. Each point represents the average of three consecutive germination tests as shown in Table II.

TABLE II

GERMINATION PERCENTAGES OBTAINED AT 15° C. FROM DELPHINIUM SEEDS STORED UNDER VARIOUS CONDITIONS FOR DIFFERENT LENGTHS OF TIME

Storage	Seed		Germination percentages after storage from 0 to 62 months following Dec 1926																		
	Annual or perennial	Crop	0	2	4	6	9	11	16	17	20	22	26	30	35	39	43	46	52	59	62*
Open room temp.	Annual	1924	27	24	30	4	8	6	4	2	4	2	0	2	0	0	0	0	0	0	0
		1925	57	55	41	46	40	41	27	24	33	13	13	9	2	1	1	1	0	0	0
		1926	72	81	76	71	62	57	66	63	60	44	46	33	5	3	0	0	0	0	0
	Perennial	1924	2	0	1	0	0	0	0	0	—	—	—	—	—	—	—	—	0	0	0
		1925	19	7	3	5	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
		1926	43	33	31	39	19	11	6	9	0	0	0	0	0	0	0	0	0	0	0
Sealed room temp.	Annual	1924		21	21	10	13	17	6	9	8	10	5	12	2	2	7	7	0	2	1
		1925		62	56	41	48	51	46	44	43	37	40	43	35	30	29	33	15	19	28
		1926		80	79	75	76	75	64	73	76	80	72	76	66	59	58	50	47	44	40
	Perennial	1924		1	0	0	0	1	0	0	—	—	—	—	—	—	—	—	0	0	—
		1925		9	8	4	2	3	1	2	2	1	1	0	0	0	0	0	0	0	0
		1926		44	31	39	29	35	27	31	22	21	12	4	4	0	0	0	0	0	0
Open 8° C.	Annual	1924		36	34	14	8	5	2	2	13	7	6	5	1	0	1	4	1	0	0
		1925		60	59	39	33	28	17	16	31	18	27	13	7	7	30	14	10	7	4
		1926		81	73	60	41	50	41	48	48	41	52	41	31	29	45	31	20	11	7
	Perennial	1924		1	0	1	0	0	0	0	—	—	—	—	—	—	—	—	0	0	—
		1925		15	8	4	0	3	0	0	1	0	0	0	0	1	0	0	0	0	0
		1926		39	31	32	12	3	4	4	3	1	1	0	0	1	0	1	0	0	0
Sealed 8° C.	Annual	1924		20	24	8	20	18	10	11	26	31	19	25	16	20	24	22	14	21	14
		1925		47	62	45	54	59	43	41	53	60	57	49	46	50	41	46	42	50	44
		1926		84	79	64	60	70	68	58	69	67	67	64	60	77	76	66	65	77	69
	Perennial	1924		0	0	0	0	0	0	0	—	—	—	—	—	—	—	—	1	0	—
		1925		5	5	10	10	9	11	4	7	5	8	3	7	6	10	6	3	0	3
		1926		37	37	34	48	39	47	40	50	45	48	52	37	38	40	46	39	33	42

TABLE II (*Continued*)

GERMINATION PERCENTAGES OBTAINED AT 15° C. FROM DELPHINIUM SEEDS STORED UNDER VARIOUS CONDITIONS FOR DIFFERENT LENGTHS OF TIME

Open −15° C.	Annual	1924		36	31	2	24	25	15	11	26	17	14	10	7	15	10	3	1	1	3
		1925		53	61	35	45	44	37	35	38	42	29	32	27	31	30	28	19	17	13
		1926		61	76	34	57	55	10	23	15	4	5	7	4	5	7	3	2	2	1
	Perennial	1924		0	0	0	0	1	0	0	—	—	—	—	—	—	—	—	0	0	—
		1925		16	9	12	10	7	11	5	10	4	6	8	6	6	4	3	4	3	1
		1926		33	41	34	47	44	45	46	43	45	35	42	48	37	33	37	38	37	35
Sealed −15° C.	Annual	1924		27	36	5	18	19	12	17	30	40	28	19	15	27	32	27	19	40	30
		1925		46	56	42	49	60	30	43	51	67	55	48	46	58	56	55	50	61	46
		1926		75	79	67	48	67	48	72	67	61	63	62	51	56	81	59	42	59	50
	Perennial	1924		0	0	1	0	0	2	0	—	—	—	—	—	—	—	—	0	0	—
		1925		17	8	5	14	16	13	12	16	12	14	0	16	18	17	17	15	18	14
		1926		37	37	36	48	42	45	45	51	53	47	51	45	53	56	57	53	42	51
Dried open −15° C.	Annual	1924		22	30	7	19	18	6	8	27	24	18	11	8	22	19	7	3	3	2
		1925		80	60	50	44	40	30	22	55	36	47	34	29	38	37	34	16	19	8
		1926		89	71	36	38	49	18	25	25	26	12	19	6	12	4	4	2	0	0
	Perennial	1924		0	0	0	0	0	0	0	—	—	—	—	—	—	—	—	0	0	—
		1925		9	12	9	8	3	4	6	6	6	5	7	11	7	4	6	5	2	2
		1926		38	18	41	37	34	38	33	49	37	36	43	40	41	42	45	31	31	30
Dried sealed −15° C.	Annual	1924		14	28	2	12	13	6	10	31	30	23	48	15	30	29	19	6	33	23
		1925		45	62	29	45	49	37	44	63	60	42	50	32	59	48	61	48	53	52
		1926		76	76	48	59	53	44	48	72	65	67	58	41	62	77	72	45	65	48
	Perennial	1924		0	0	0	0	0	0	0	—	—	—	—	—	—	—	—	1	1	—
		1925		2	6	7	10	5	14	12	17	13	17	15	12	19	18	15	12	9	15
		1926		36	28	45	45	36	41	45	41	42	43	58	48	44	50	53	44	53	55

* 500 seeds each used in this test.

the end of 39 months the germination is 0, 1.7, and 4.1 per cent of that of the original test for 1924, 1925, and 1926 seeds respectively. On the other hand, if the seeds are kept in sealed containers at room temperature, the viability of the 1924, 1925, and 1926 seeds at the end of 39 months of storage is 7.4, 52.6, and 81.9 per cent of the original. Fairly good germination was obtained from 1925 and 1926 seeds stored sealed at room temperature even after 62 months of storage.

When the seeds are stored at 8° C. or −15° C. the beneficial effect of sealing is still apparent. However, the longevity of the seeds at these temperatures is not so dependent on sealing as is the case with room temperature storage (Fig. 1 and Table II). Open storage at low temperatures is far superior to open storage at room temperature. Sealed storage at room temperature however, is equal to, if not actually better than open storage at 8° C. or −15° C. (Table II).

Except for the 1926 crop, all seeds kept best at −15° C. whether in open or sealed containers. This behavior of the 1926 seeds may be due to the fact that the fresh seeds had not dried out previous to storage to the extent that the one-year and two-year-old seeds had done. The additional moisture might have caused injury when the seeds were in a freezing temperature. In all cases, however, seeds stored at 8° C. retained their vitality better than those stored at room temperature.

From a study of Table II it becomes evident that the temperature at which the seeds are stored does not have such a marked effect if the containers are sealed. On the other hand, if the containers are to be left open, it is very important that the seeds be kept in a cool place.

Storage of Perennial Seeds

Under unfavorable conditions of storage perennial seeds lose their vitality much more quickly than seeds of the annual plant. Seeds of the 1924 crop were practically dead when they were received. Except at −15° C., deterioration of the one-year-old and fresh seeds in open storage was very rapid. A sharp decline in viability made the seeds practically worthless after 9 to 16 months (Table II).

In their response to different storage conditions, however, perennial seeds were similar to annual seeds. Sealed storage was much more effective than open storage at room temperature and at 8° C. However, at −15° C., which was decidedly the most favorable storage temperature for perennial seeds, the advantage gained by sealing was negligible up to 30 months of storage after which seeds from sealed containers maintained higher germination percentages.

TABLE III

GERMINATION AND SEEDLING PRODUCTION OF STORED AND FRESH DELPHINIUM SEEDS

Storage	Seed		Storage after Dec. 1926			
			39 months		62 months	
	Annual or perennial	Crop	Per cent germination at 15° C.	Seedling production in greenhouse at 21° C.	Per cent germination at 15° C.	Seedling production in greenhouse at 15° C.
Sealed room temp.	Annual	1924	2	1	1	0
		1925	30	1	28	8
		1926	59	22	8	15
	Perennial	1925	0	0	0	0
		1926	0	1	0	0
Open 8° C.	Annual	1924	0	1	0	0
		1925	7	0	4	1
		1926	29	8	7	1
	Perennial	1925	1	0	0	0
		1926	1	1	0	0
Sealed 8° C.	Annual	1924	20	3	14	10
		1925	50	1	44	43
		1926	77	32	69	57
	Perennial	1925	6	0	3	2
		1926	38	21	42	27
Open −15° C.	Annual	1924	15	0	3	0
		1925	31	2	13	7
		1926	5	2	1	0
	Perennial	1925	6	3	1	1
		1926	37	38	35	24
Sealed −15° C.	Annual	1924	27	0	30	15
		1925	58	1	46	43
		1926	56	25	50	58
	Perennial	1925	18	6	14	10
		1926	53	39	51	43
Dried open −15° C.	Annual	1924	22	0	2	1
		1925	38	0	8	1
		1926	12	2	0	0
	Perennial	1925	7	5	2	1
		1926	41	37	30	20
Dried sealed −15° C.	Annual	1924	30	0	23	14
		1925	59	0	52	47
		1926	62	23	48	59
	Perennial	1925	19	8	15	9
		1926	44	35	55	47
Open laboratory	Annual	*	69	0	69	76
		*	56	0	—	—
	Perennial	*	79	63	96	84
		*	75	63	—	—

Effect of Drying

Samples of seeds of both annual and perennial delphinium were dried over quicklime for 10 days. These dried seeds were then stored open and sealed at $-15°$ C. Drying the seeds in this manner had no appreciable effect on the keeping quality at this temperature (Table II).

Seedling Production in the Greenhouse

Plantings made in the greenhouse after 39 months of storage indicated that seedling production was not so great as the percentage of germination

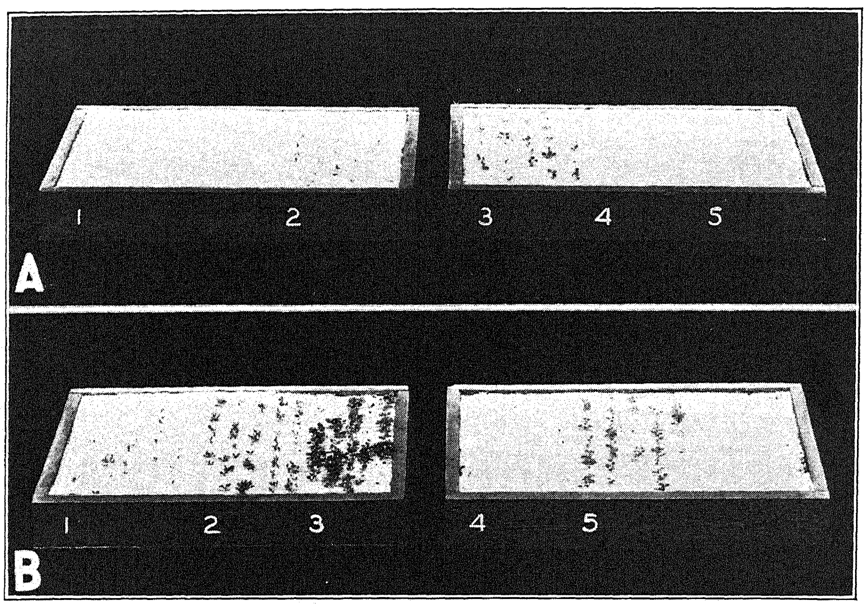

FIGURE 2. Seedling production in the greenhouse (15° C.) from delphinium seeds stored sealed for 62 months following December 1926. 1. annual 1924 crop; 2. annual 1925 crop; 3. annual 1926 crop; 4. perennial 1925 crop; 5. perennial 1926 crop. A. stored at room temperature; B. stored at 8° C.

obtained at 15° C. at the same time (Table III). It will be noted, however, that in the case of the 1926 perennial seeds in the more favorable storage conditions (sealed at 8° C. or $-15°$ C.), the proportion of the number of seedlings produced to the germination percentages in the oven is essentially the same as for fresh seeds.

Peculiarly, the fresh annual seeds produced no seedlings in the greenhouse in spite of the fact that they germinated to the extent of 69 and 56 per cent at 15° C. The annual seeds from storage were also less capable of seedling production than the perennials. The explanation of this phenom-

enon is that the greenhouse temperature (21° C.) was too high for the best germination of annual seeds, which are apparently more sensitive to high temperatures than perennial seeds (Table I).

The plantings made after 62 months of storage were kept in a greenhouse with a temperature of about 15° C. Table III indicated a marked improvement in seedling production from annual seeds and a less marked increase in the number of perennial seedlings. These tests showed very clearly that seeds from the favorable storage conditions were still capable

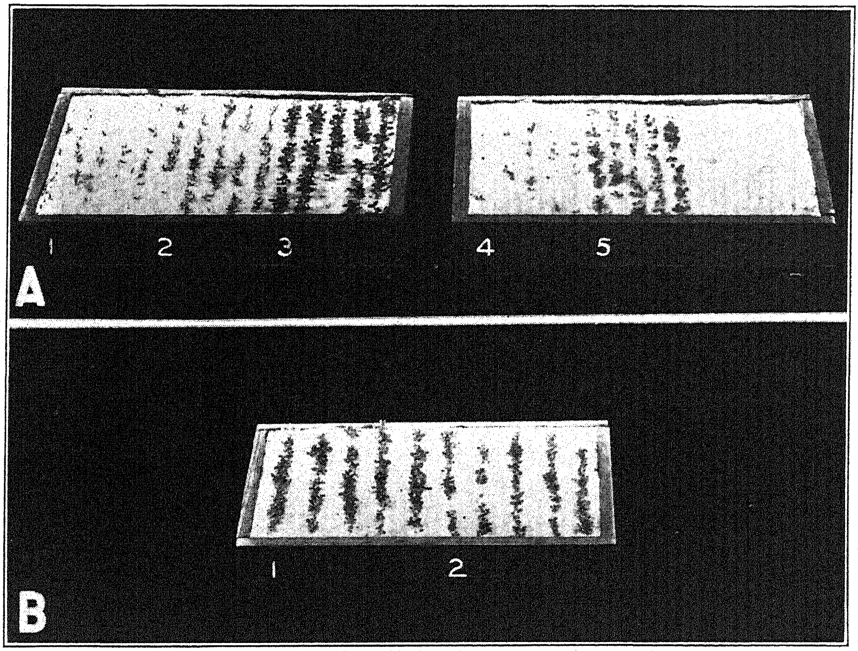

FIGURE 3. Seedling production in the greenhouse (15° C.) from delphinium seeds A. Stored sealed at −15° C. for 62 months following December 1926. 1. annual 1924 crop; 2. annual 1925 crop; 3. annual 1926 crop; 4. perennial 1925 crop; 5. perennial 1926 crop. B. Fresh seeds. 1. annual 1931 crop; 2. perennial 1931 crop.

of good seedling production in the greenhouse and that this production compared favorably with the germination obtained on filter paper at 15° C.

Figures 2A and B, and 3A show seedling production from annual and perennial seeds which had been stored 62 months in sealed vials at room temperature, 8° C., and −15° C. The seedling production from fresh seeds is also shown (Fig. 3B). It will be seen that in each case the 1924 seeds showed least vigor both in number of seedlings produced and rapidity of growth. The vigor of the 1925 seeds occupied an intermediate position between the 1924 and 1926 seeds. The vigor of the latter from cold, sealed

storage compared favorably with that of fresh seeds (Fig. 2B and Fig. 3A and B).

The results of the greenhouse plantings of delphinium seeds from favorable storage conditions are in opposition to the statements of Franck (6) and Haack (7) that the decrease in seedling production is more rapid than the fall in germination percentage. In cases of unfavorable storage conditions, however, the contention of these investigators that seeds are still capable of germination after they can no longer produce a good seedling stand is confirmed.

In order to preserve the seedling-producing power of delphinium seeds, then, it is only necessary to store them properly.

SUMMARY

1. Storage experiments with two-year-old, one-year-old, and fresh seeds of annual and perennial delphinium were begun in December 1926.

2. Germination tests at the beginning and after 16, 35, and 39 months of storage indicated that a constant temperature of 15° C. and a daily alternating temperature of 10° and 20° C. were most favorable for germination.

3. Samples were stored open and in sealed glass vials at room temperature, 8° C., and −15° C. Germination tests to determine the viability were made at intervals of from one to seven months. Results of these tests indicate:

a. At any temperature and especially at room temperature sealed storage is much more effective than open storage. This advantage becomes more marked as the storage period lengthens.

b. For keeping the seeds viable, 8° C. and −15° C. are superior to room temperature, especially in the case of open storage.

c. Seeds of perennial delphinium deteriorate more rapidly under unfavorable storage conditions than seeds of the annual plant.

4. Greenhouse tests of seeds from favorable storage conditions showed the ability to produce a good stand of seedlings even after 62 months of storage.

5. In storing delphinium seeds, sealed containers should be the primary consideration. If, in addition, these containers are stored in a cool place a more nearly perfect condition for maintenance of viability could be obtained.

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EFFECT OF ILLUMINATING GAS ON THE LILY, NARCISSUS, TULIP, AND HYACINTH

A. E. Hitchcock, William Crocker, and P. W. Zimmerman

Previous papers concerning the effect of illuminating gas or ethylene on plants contain few references to liliaceous species. Doubt (3) stated that several varieties of tulips and hyacinths responded alike to 10,000 parts of illuminating gas (containing 4 per cent ethylene) to 1,000,000 parts of air when the plants were treated for four days. The flower buds were injured and the tips of young leaves rolled up. A concentration of 4000 p.p.m. failed to produce these responses. Crocker (2) described the stunting effect of illuminating gas on Easter lilies grown in a range of commercial greenhouses. In one house where the presence of gas was established, the lilies were from three to four inches shorter than those in another house where no signs of gas could be detected. Both lots of plants were of the same age and they were grown under similar conditions except with respect to the amount of illuminating gas present. Zimmerman, Crocker, and Hitchcock (4) reported that a concentration of one part of illuminating gas to 20,000 parts of air killed the flower buds of *Lilium harrisii*, but that the leaves were resistant to a concentration of one part of gas to 1000 parts of air.

The present paper describes the effects particularly of illuminating gas on the growth and flowering of the lily, narcissus, tulip, and hyacinth. Ethylene was used in some cases since this is the most effective constituent in illuminating gas which causes injury on many plants. The work reported in this paper constitutes one phase of the general project concerning the effect of gases on plants.

METHOD

Plants were exposed to gas in Wardian cases 3 ft. x 3ft. x 7ft. or in cans of 64 liter capacity. The cases were used in most experiments in order that the plants would receive light during long periods of treatment. The time of exposure of the plants to gas was usually three days or more. Measured amounts of gas were obtained by the displacement of known volumes of water in glass containers. After being placed in the case with the plants, the gas was released from the containers and the door of the case was quickly closed and sealed with plasteline clay. When the period of treatment was more than three days, the cases were opened at the end of each two or three days, aired out, and then the plants were given a fresh supply of gas. Control plants were placed in similar cases to which no gas was added. Growth measurements were made immediately before and after treatment except for those tests which required an additional measurement on the second day of a four-day treatment. The maximum and minimum concentrations of gas tested for each variety of plant are given in Table I.

TABLE I

MAXIMUM AND MINIMUM CONCENTRATIONS OF ILLUMINATING GAS TO WHICH EACH VARIETY OF PLANT WAS SUBJECTED

Variety of plant	No. plants used	No. of tests	Concentration of illuminating gas	
			Maximum	Minimum
Lilium longiflorum Thunb. var. Giganteum	30	9	1 to 100	1 to 40,000
" " " " *eximum* Nichols (*L. harrisii* Carr.)	15	11	1 to 100	1 to 40,000
Lilium speciosum Thunb. var. Rubrum	32	19	1 to 1000	1 to 100,000
Narcissus tazetta L. var. Paper White	28	11	1 to 100	1 to 40,000
" *odorus* L. var. Campernelle	15	14	1 to 100	1 to 40,000
" sp. var. King Alfred	8	6	1 to 5000	1 to 20,000
" sp. var. Mrs. Langtry	14	5	1 to 100	1 to 20,000
" sp. var. Laurens Koster	10	3	1 to 10,000	1 to 20,000
Tulipa gesneriana L. var. Argo	22	6	1 to 75	1 to 100
" " " " Idyll	23	4	1 to 75	1 to 100
" " " " Inglescombe Yellow	11	4	1 to 100	1 to 100
" " " " La Fiancee	16	3	1 to 75	1 to 100
" " " " Nectar	12	2	1 to 100	1 to 100
" " " " William Copeland	95	17	1 to 75	1 to 20,000
" sp. (variety unknown)	13	10	1 to 100	1 to 40,000
Hyacinthus orientalis L. var. Bismark	50	21	1 to 75	1 to 40,000
" " " " Marconi	30	6	1 to 75	1 to 100
" " " " L'Innocence	6	3	1 to 100	1 to 10,000

Analyses of the illuminating gas from the mains of the Westchester Lighting Company, Yonkers, New York, indicated 5.6 per cent illuminants. According to analyses of illuminating gas given in the Gas Chemists' Handbook (1, p. 133, 136), ethylene constitutes 60 to 70 per cent of the illuminants. On this basis the illuminating gas used in our experiments contained approximately 3 per cent ethylene. The pure ethylene used in these experiments was purchased from the United States Industrial Chemical Company, New York City. Analyses of this gas showed 97.2 per cent absorption by a saturated solution of bromine (5).

RESULTS OF EXPERIMENTS

LILIUM

Retardation of growth in gas. Growth measurements for the varieties Giganteum *eximium* (hereafter referred to as Harrisii), and Rubrum are given in Tables II, III, and IV respectively. Values for the percentage of retardation of growth represent the percentage difference between the growth increments of the control and treated plants. For example, in Table II, column three, lines three and four, the difference between the values for the control (3.8) and for the treated plant (2.5) is 1.3 centimeters or 34 per cent, which represents the retardation in growth due to treatment. Expressed as the rate of growth during treatment instead of as the percentage of retardation, the value 34 would then become 66 per cent.

TABLE II

EFFECT OF ILLUMINATING GAS ON THE GROWTH AND FLOWERING OF THE GIGANTEUM LILY

Treatment	No. days treated	Elongation during treatment		Response after treatment
		Increase in height cm.	Percentage retardation of growth during treatment	Condition of, or day, buds appeared
Control	3	5.1	—	28th
Illum. gas 1 to 10,000	"	0.0	100	"
Control	"	3.8	—	"
Illum. gas 1 to 20,000	"	2.5	34	"
Control	"	7.6	—	"
Illum. gas 1 to 100	"	0.0	100	"
Control	"	5.1	—	"
Illum. gas 1 to 40,000	"	1.3	75	"
Control	7	7.0	—	12th
Illum. gas 1 to 20,000	"	0.0	100	Aborted on 31st
Control	"	8.0	—	12th
Illum. gas 1 to 20,000	"	1.0	88	Aborted on 31st
Control	4	2.0	—	15th
Illum. gas 1 to 100	"	0.0	100	29th
Control*	3	2.0	—	—
Illum. gas 1 to 10,000*	"	2.0	0	Dead on 23rd day

* These plants had young flower buds at the time of treatment.

The average percentage of retardation is 75 for Giganteum (Table II), 89 for Harrisii (Table III), and 43 for Rubrum (Table IV). The growth of Giganteum and Harrisii plants was retarded by 1 to 40,000, the lowest concentration of illuminating gas used. Some of the Rubrum plants were retarded in growth by ethylene in a concentration of 1 to 1,000,000. No definite relation exists between the values for the amount of retardation and the concentration of gas used. For example, illuminating gas in concentra-

tions of 1 to 100 and 1 to 20,000 caused a cessation of growth of the Giganteum lily (Table II).

The Giganteum plants referred to in Table II were from 16 to 23 centimeters tall, except the two having flower buds which were 27 to 30 centimeters in height. Harrisii plants (Table III) bearing no flower buds were from 36 to 45 centimeters tall, and those with flower buds were 58 to 71 centimeters high. The heights of Rubrum plants are given in Table IV.

TABLE III

EFFECT OF A THREE-DAY EXPOSURE TO ILLUMINATING GAS ON THE GROWTH AND FLOWERING OF THE HARRISII LILY

Concentration of gas	Presence of, or relative size of buds when treated	Elongation during treatment		Response after treatment	
		Increase in height cm.	Percentage retardation of growth during treatment	Condition of, or day, buds appeared	Flowering
Control	Small	4.1	—	Normal	Completed on 41st day
1 to 20,000	"	1.1	73	Shriveled	None
1 to 5000	"	0.8	81	"	"
Control	None	2.3	—	52nd day	Started on 84th day
1 to 20,000	"	0.0	100	" "	Started on 85th day
1 to 5000	"	0.0	100	35th day	Started on 71st day
Control	Small	3.5	—	Normal	Completed on 39th day
1 to 40,000	"	0.5	86	All killed	None
1 to 1000	"	1.0	71	" "	"
Control	None	2.0	—	43rd day	Started on 77th day
1 to 4000	"	0.0	100	" "	Started on 79th day
1 to 1000	"	0.0	100	—	Started on 57th day

Modified leaf growth. Young leaves of the Rubrum lily frequently showed epinasty (an arched downward bending) when exposed to illuminating gas. Curling (epinasty) of the tips of two leaves occurred on a Rubrum plant exposed three days to illuminating gas in a concentration of 1 to 5000. In a concentration of 1 to 500 the actively growing leaves rolled inward along the margins or they became arched at the tip. Both of these responses were hyponastic, that is, the under side of the leaf grew faster than the upper side. The arched type of hyponasty caused the leaves in the terminal growing bud to curve inward toward one another instead of out-

ward as they would do normally in the absence of gas. Twisted leaves were not observed on any of the Rubrum plants.

Leaves on the first lot of Giganteum plants showed no bending when exposed to gas that was essentially different from that on the leaves of control plants (Fig. 1). Twisted leaves were observed on both the treated and control plants of Giganteum and Harrisii. The second lot of Giganteum plants showed marked curling of many of the tip leaves after a 24-hour exposure to 1 to 10,000 illuminating gas, but the curl was not permanent.

TABLE IV

EFFECT OF A THREE-DAY EXPOSURE TO ILLUMINATING GAS OR TO ETHYLENE ON THE GROWTH OF THE RUBRUM LILY

Treatment	Height before treatment cm.	Height after treatment cm.	Increase in height cm.	Percentage retardation of growth during treatment
Control	30	36	6	—
Illum. gas 1 to 10,000	37	40	3	50
Control	27	37	10	—
Illum. gas 1 to 10,000	24	26	2	80
Control	18	23	5	—
Illum. gas 1 to 20,000	20	24	4	20
Control	72	75	3	—
Ethylene 1 to 1,000,000	90	93	3	0
Control	85	88	3	—
Ethylene 1 to 1,000,000	100	101	1	66

Although there was some twisting on the young leaves, the most consistent response was epinasty in the form of arched bending. This particular lot of plants was forced into active growth by an immediate transfer from a low to a high temperature. Young leaves on all of the actively growing Giganteum plants showed simple epinasty (arched bending) and twisting as a result of exposure to illuminating gas.

Effect of gas on flowering. Young flower buds on Harrisii plants were usually killed by concentrations of illuminating gas of 1 to 40,000 or higher (Table III, columns five and six). Some growth of the flower buds occurred in a concentration of 1 to 20,000, but these buds aborted soon after re-

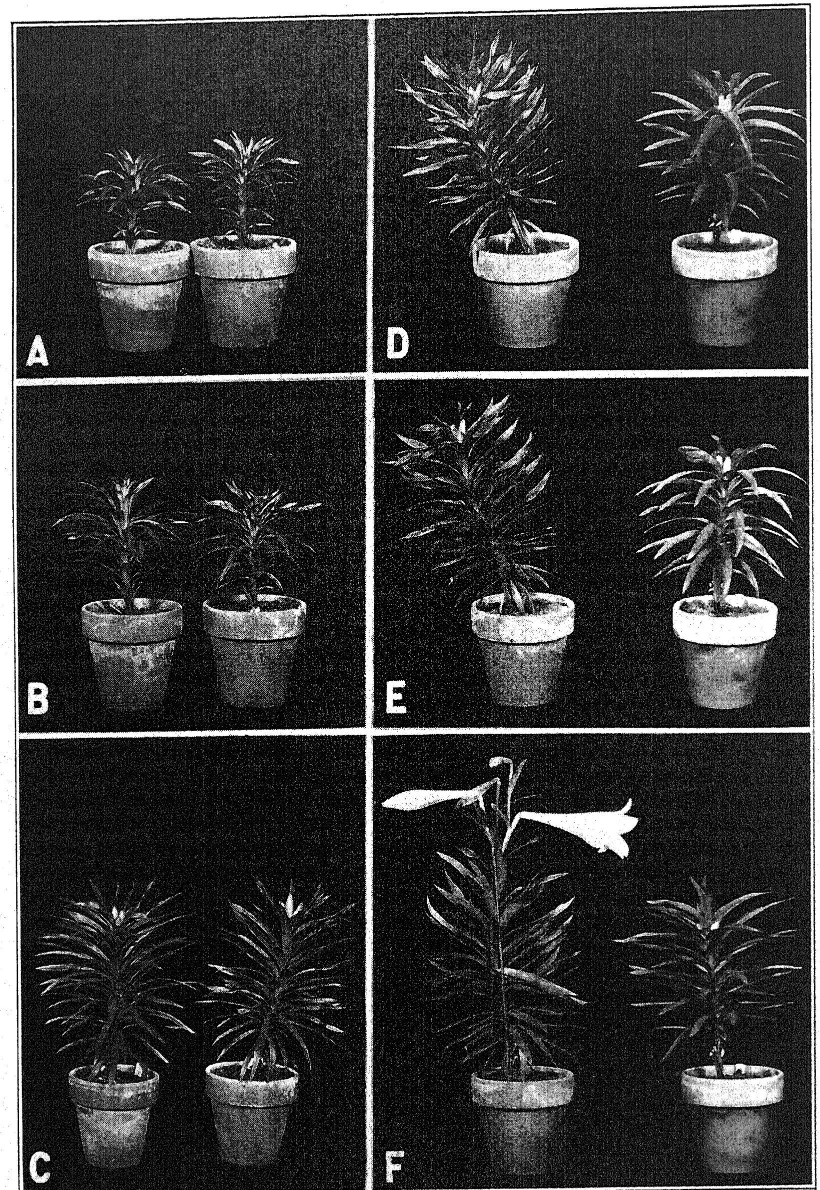

FIGURE 1. Effect of a three-day exposure to 1 to 10,000 illuminating gas on the Giganteum lily with and without flower buds at the time of treatment. In each case the control plant is on the left. A. No flower buds, and D with flower buds, as the plants appeared before treatment. The same plants are shown in B and E as they appeared after removal from gas. In C and F the same plants are shown 34 and 23 days respectively after removal from gas. In the series D, E, F the young flower buds were killed by the gas. When no flower buds were visible at the time of treatment, as in A, B, C, the gas did not alter the normal course of flower bud formation and subsequent development after removal from treatment.

moval from gas. If no flower buds were present on Giganteum at the time of treatment as shown in Figure 1 A, a concentration of 1 to 10,000 illuminating gas did not prevent the formation of flower buds as shown for a later date in Figure 1 C. Flowering for the plants shown in Figure 1 D was prevented by treatment with gas, since flower buds were visible at the time of treatment. The Giganteum and Harrisii lilies responded alike when given the same treatments with illuminating gas.

Flower buds of the Rubrum lily were sometimes killed when exposed three days to 1 to 20,000 illuminating gas. No injury resulted when the flower buds were exposed to ethylene in a concentration of 1 to 1,000,000, which is equivalent to 1 to 30,000 illuminating gas.

NARCISSUS

Retardation of growth. There were no marked varietal differences with respect to leaf elongation during treatment, the average percentage of retardation being 69 for the five varieties of narcissus. The growth measurements for the Paper White and Laurens Koster are given in Table V. Low concentrations usually produced less retardation than high concentrations. There was slight but definite retardation of leaves in 1 to 20,000 illuminating gas and marked retardation at higher concentrations (Figs. 2, 3, and 4, and Table V). Leaves of the Paper White were slightly retarded in 1 to 40,000 illuminating gas.

The retardation in growth of the flower stalks during treatment was usually equal to and sometimes greater than that for leaves on the same plant. In a concentration of 1 to 20,000 or higher, the flower stalks of Paper White and Laurens Koster never equaled the height of those on control plants, even though flowering occurred (Figs. 2 and 4). Likewise the flower stalks of Paper White were slightly retarded in growth when previously treated for four days in 1 to 40,000 illuminating gas.

Modified leaf growth. Curvatures ranging from a slight declination to a right angle bend were produced on young leaves of Paper White, Laurens Koster, and Mrs. Langtry narcissus as a result of exposure to illuminating gas in concentrations of 1 to 10,000 or higher (Figs. 2, 3, and 4). Bending did not occur with reference to a particular side of the leaf, since in some cases all leaves bent in the same direction (Fig. 5 D). Leaves taller than 15 centimeters usually showed no bending as a result of treatment. After removal from gas, a partial or complete recovery of the leaves sometimes occurred, particularly if the period of treatment was not more than two days.

Marked curling of the tips of young leaves occurred on the Paper White (Fig. 2 A) but not on the other varieties of narcissus. The area inclosed by the curl was circular in outline. This type of curvature was permanent even though the leaf continued to grow (Fig. 2 B) and is different in this respect from the temporary curl on the leaves of the Giganteum and Rubrum lilies. The older leaves did not curl.

TABLE V

EFFECT OF ILLUMINATING GAS ON THE GROWTH AND FLOWERING OF TWO VARIETIES OF NARCISSUS

Variety	Concentration of gas	Growth during treatment: Increase in height cm.		Growth during treatment: Percentage retardation of growth during treatment		Response after treatment	
		Leaf	Flower stalk	Leaf	Flower stalk	Flower buds*	Flowering
Paper White	Control 1	7.0	15.2	—	—	Normal	All flowers open 1st day
	Control 2	8.3	10.8	—	—	Normal	" " " 6th "
	1 to 20,000	6.3	4.4	17	66	Slight injury	1 flower partly open 6th day
	1 to 10,000	1.3	0.0	82	100	All 3 dead	None
	1 to 1,000	0.0	0.6	100	95	All 5 dead	"
	1 to 500	1.9	0.6	88	95	—	"
Laurens Koster	Control	7.6	15.0	—	—	Normal	In flower 1st day
	1 to 20,000	2.5	10.2	67	36	Opened prematurely	Flower small, shriveled
	Control	—	24.1	—	—	Normal	In flower 1st day
	1 to 20,000	—	10.2	—	58	Opened prematurely	Flowers small, shriveled
	Control	8.9	24.1	—	—	Normal	In flower 1st day
	1 to 10,000	1.3	3.2	85	87	Growth retarded	None fully opened
	Control	—	25.0	—	—	Normal	In flower 1st day
	1 to 10,000	—	3.8	—	85	Growth retarded	None fully opened

* Refers to flower shoot and not to individual buds on the same shoot.

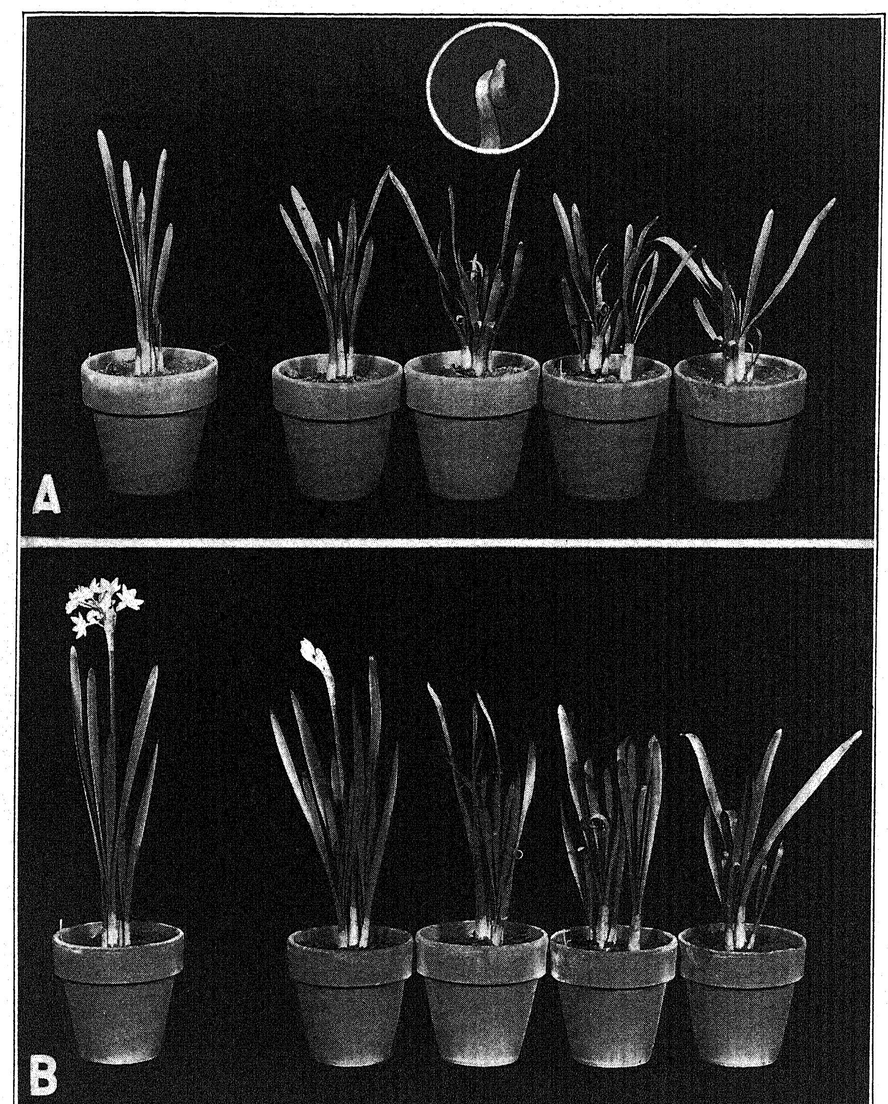

FIGURE 2. The effect of a three-day exposure to different concentrations of illuminating gas on the growth and flowering of the Paper White narcissus. From left to right: Control, 1 to 20,000, 1 to 10,000, 1 to 1000, and 1 to 500. A. Photographed immediately after treatment. The young leaves of the plants given concentrations of 1 to 10,000 or higher show a retarded growth and marked curling of young leaves. An enlargement of a curled leaf is shown in the insert. In a concentration of 1 to 20,000 there was no curling and the growth of leaves and flower stalks was less retarded than at higher concentrations. B. The same plants six days after removal from gas, showing the permanency of the curls, and the effect of gas on flowering.

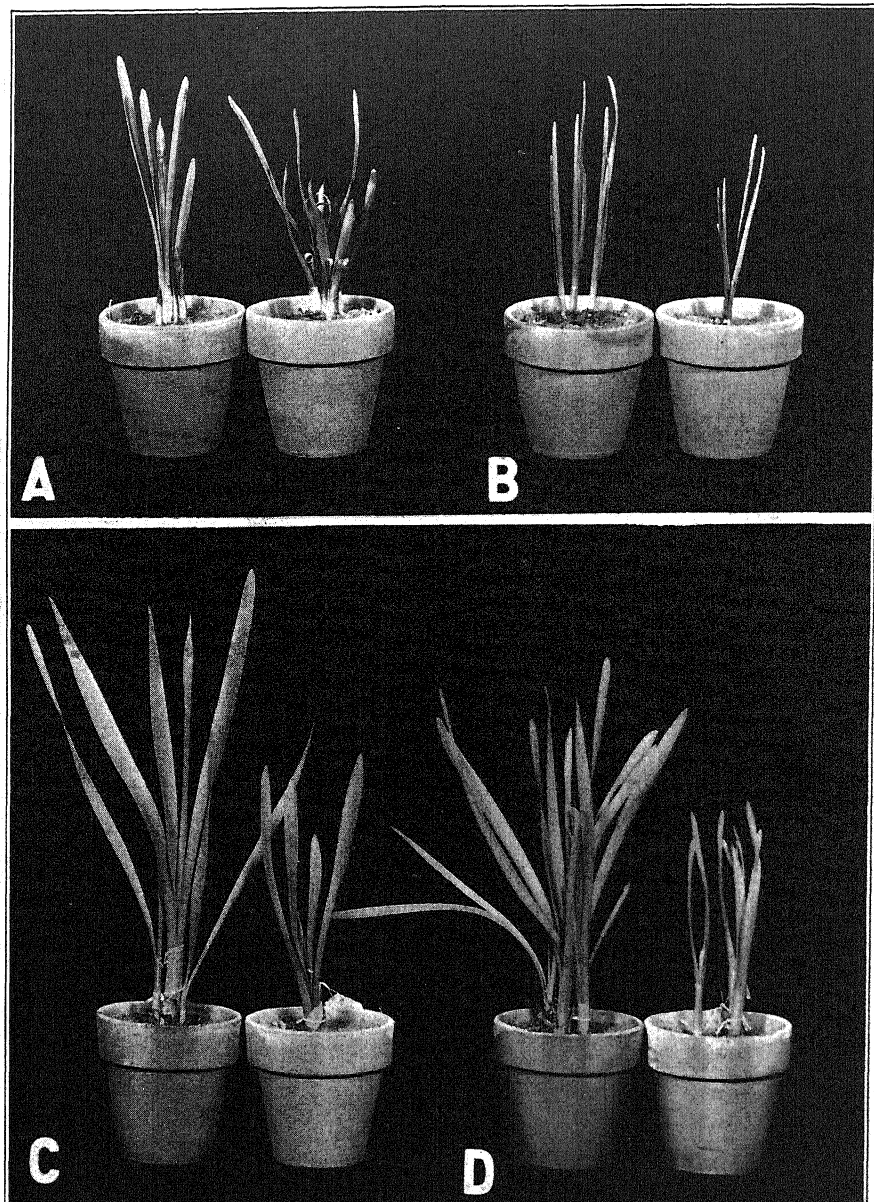

FIGURE 3. Effect of a three-day treatment with 1 to 10,000 illuminating gas on the elongation and modified growth of the leaves of four varieties of narcissus. A, Paper White; B, Campernelle; C, King Alfred; and D, Mrs. Langtry. A marked curling of young leaves occurred only on the Paper White as a result of gas treatment.

The leaves of the variety Mrs. Langtry showed a looped type of curl (Fig. 5 D) which was different both in shape and in manner of formation from the curl on Paper White leaves (Fig. 2). Whereas the curl on Paper White leaves was less than one centimeter in diameter and involved only the tip, the loop formed by Mrs. Langtry leaves was much larger in diameter (one to three centimeters) and the bending began from three to six cen-

FIGURE 4. The effect of two different concentrations of illuminating gas on the growth and flowering of the Laurens Koster narcissus. Photographed three days after removal from a three-day treatment in gas. From left to right: Control, 1 to 20,000, control, and 1 to 10,000. Note the relative effect of the two concentrations on flowering and the growth of leaves.

timeters away from the tip. The curl on Paper White leaves was circular in shape, but the loop on Mrs. Langtry leaves was arched at the top and V-shaped at the base (Fig. 5 D). Paper White leaves taller than eight centimeters usually did not curl. The loop response on Mrs. Langtry plants usually occurred on leaves which were 8 to 16 centimeters in height and re-

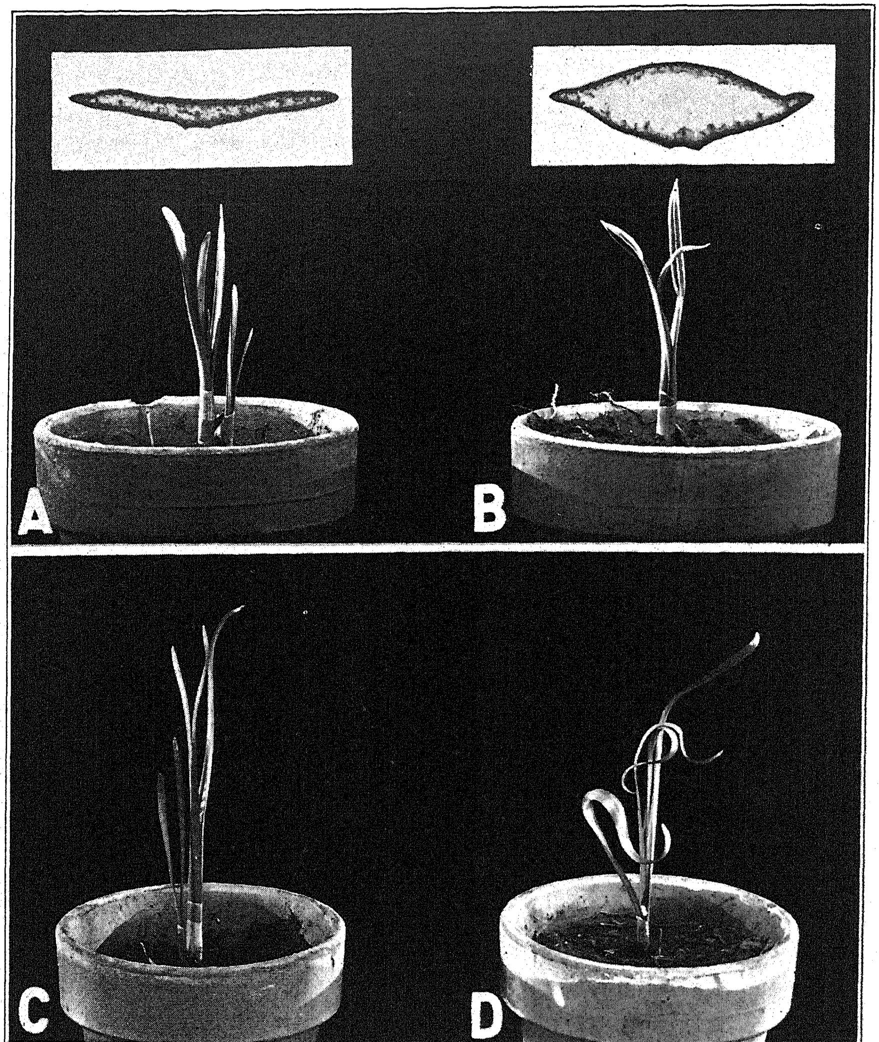

FIGURE 5. Effect of a three-day exposure to 1 to 10,000 illuminating gas on the leaves of Mrs. Langtry narcissus. Control (A) and treated (B) plants as they appeared immediately after treatment. A cross-section of a leaf from the control plant is shown in A. A cross-section of an inflated leaf from the treated plant is shown in B. Inserts A and B are magnified 5 times. The swelling response is specific for this variety of narcissus. The plants in C and D were photographed two days after removal from gas. The leaf with the largest double bend in D was inflated when removed from gas, but the swelling had disappeared at the end of the second day.

mained as a permanent distortion. Leaves taller than 16 centimeters sometimes bent but usually did not form loops, and the leaves less than eight centimeters usually made a right angle bend at or near the middle of the leaf. A double bending response (Fig. 5 D) was frequently associated with the formation of a loop. In this case just previous to the closing of the loop, the tip of the leaf bent upward in a direction opposite to the original curvature, so that there was both an epinastic and a hyponastic response on the same leaf. Some of the leaves with the double bend formed a figure eight as shown in Figure 5 D.

Young leaves of Mrs. Langtry swelled to a thickness of two to six millimeters (Fig. 5 B) when subjected to illuminating gas in concentrations of 1 to 10,000 or higher. The length of the inflation was usually about three centimeters, beginning at the tip but in some cases it was 15 centimeters long. The cavity thus formed was filled with cell sap which showed an abundance of reducing substances and the presence of masses of calcium oxalate crystals. Cross-sections of the cavity (Fig. 5 B) showed that the longitudinal split occurred approximately through the middle of the loose central tissue. The presence of an inflation did not prevent the leaf from bending and in many cases swelling occurred after bending had started. Although the tips of non-inflated leaves sometimes turned yellow after treatment with high concentrations of illuminating gas, the inflated leaves usually turned yellow first. The inflation usually disappeared one to three days after removal from gas.

Plants of the varieties King Alfred and Campernelle narcissus were not subjected to treatment when the leaves were younger than is shown in Figure 3 B and C. Older leaves of these two varieties showed no curvatures when treated with illuminating gas.

Effect of gas on flowering. Flower buds on the Paper White and Laurens Koster were injured by 1 to 20,000 illuminating gas or higher concentrations (Figs. 2 and 4). A concentration of 1 to 40,000 did not prevent the flowering of Paper White, but the flowers did not last as long as those on the control plants. In concentrations higher than 1 to 20,000 the young flower buds were usually killed, but some of these plants later produced new flower stalks which developed normally. Mature flower buds generally opened more quickly during treatment; nearly mature buds were retarded in opening; and young buds were severely injured or killed. Abnormal flowering was characterized by premature or delayed opening, fewer flowers, smaller size, the failure to open fully, or premature withering. Injured petals on all narcissus flowers were of a transparency similar to tissue paper, and they always shriveled or withered prematurely as shown for Laurens Koster in Figure 4. Flowers on control plants of Paper White lasted two or three days longer than those on plants subjected to illuminating gas.

TULIPA

Retardation of growth. Growth measurements for the William Copeland tulip are given in Table VI. The average value for the percentage of retardation is 87. Leaves of these plants were 14 to 17 centimeters high at the time of treatment.

TABLE VI

EFFECT OF A THREE-DAY EXPOSURE OF 1 TO 500 ILLUMINATING GAS ON THE GROWTH OF THE WILLIAM COPELAND TULIP

Treatment	Height of leaves before treatment cm.	Gain in height of leaves during treatment cm.	Percentage retardation of growth during treatment
Control	14.0 13.0	6.3 4.4	— —
Control	15.2 14.6	6.3 4.8	— —
1 to 500	15.9 15.2	0.6 0.6	90 86
1 to 500	13.0 13.0	1.3 0.3	79 94
Control	14.3 12.7	4.8 3.2	— —
Control	15.9 16.5	5.1 4.4	— —
1 to 500	15.2 15.2	1.9 0.6	60 81
1 to 500	13.3 13.3	1.9 0.6	63 86

The varieties Argo, Nectar, Idyll, and La Fiancee were exposed four days to 1 to 100 illuminating gas when the leaves were four to six centimeters high. These plants were growing slowly at the time of treatment. The average percentage of retardation was 50 during the first two days and 72 during the last two days of treatment. Flower stalks were retarded in growth by all concentrations of gas used, ranging from 1 to 100 to 1 to 20,000.

Modified leaf growth. The edges of some of the young leaves of the Argo and La Fiancee tulips rolled inward when exposed four days to 1 to 100 illuminating gas. This response was most pronounced on plants in which the first and second leaves were in the process of unfolding. Usually the second leaf failed to unfold, although elongation continued after removal from gas. Some treated plants, however, did not show this response. Leaves of the varieties Idyll, Inglescombe Yellow, and Nectar frequently showed

inward rolling of the edges under normal conditions and in the absence of gas. For these three varieties, therefore, it was difficult to determine whether a pronounced rolling on treated leaves was due entirely to gas.

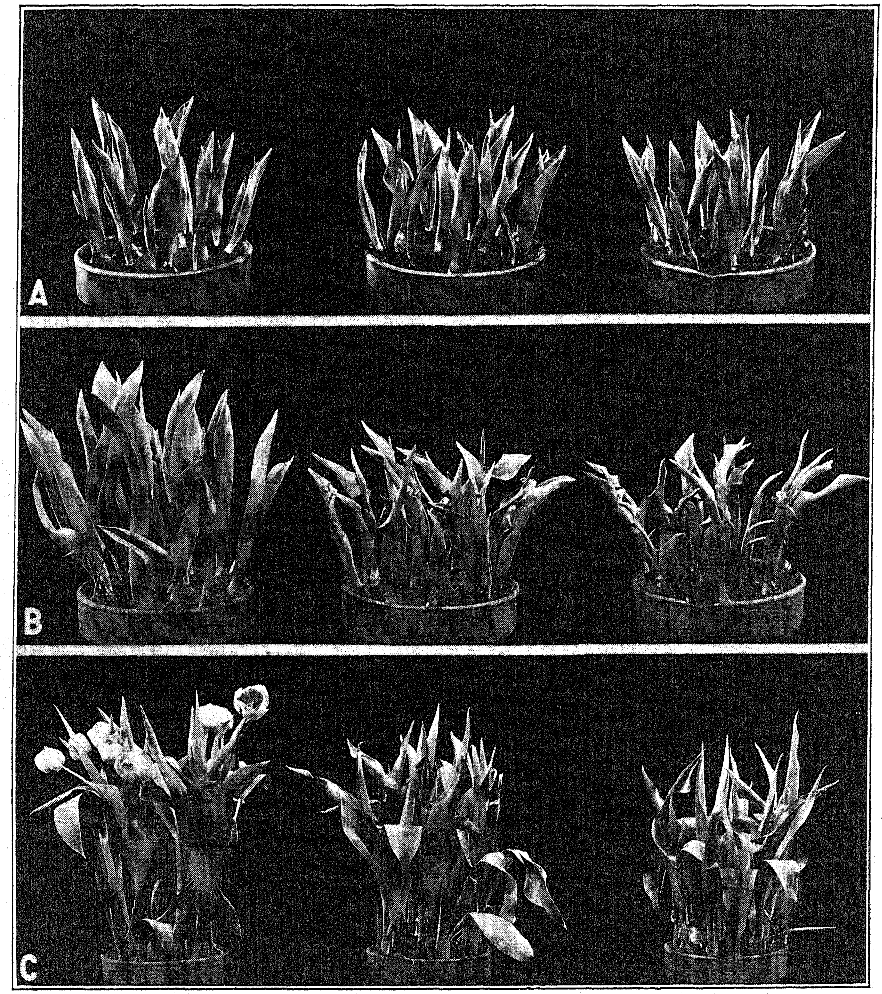

FIGURE 6. Effect of illuminating gas on young actively growing leaves of the William Copeland tulip. The same plants were photographed at three different times. From left to right: Control, 1 to 10,000, and 1 to 100. A. Appearance of plants immediately before treatment; B. The same plants after being exposed three days to gas. Illuminating gas has caused arched bending, an irregular inward rolling, and a retarded growth of the leaves; C. The same plants 15 days after removal from gas. Leaves on the treated plants have not unrolled completely and they still show wavy margins. The treated plants have resumed a rapid rate of growth and they show nearly complete recovery from the arched bending response, but all of the flower buds were killed.

One lot of William Copeland tulips was removed from the cold frames on February 18 and another lot on February 28. After two days in a greenhouse, plants of the first lot were 12 to 15 centimeters high. None of the leaves had unrolled at the time the plants were exposed for three days to 1 to 10,000 illuminating gas. Leaves on 9 of the 12 treated plants showed distortions which did not appear on leaves of the 10 control plants. The distortions consisted of an inward rolling along the margins of the leaf or an arched bending of the upper part of the leaf. Irregular inward rolling produced a wavy margin on some of the leaves. All treated plants were noticeably stunted and the leaves failed to unroll as they did on the control plants.

The second (February 28) lot of William Copeland plants was divided into several groups. One group of 12 plants was subjected three days to 1 to 10,000 illuminating gas and one group to a concentration of 1 to 100. Two groups of 12 plants each were used as controls.

At the end of 24 hours the first group of plants to be placed in 1 to 100 illuminating gas showed unmistakable distortion of the leaves. Inward rolling of the margins and arched bending occurred on the first and second leaves. The plants in 1 to 10,000 illuminating gas showed only slight bending and inward rolling. Only 1 of the 24 control plants showed an inward rolling response at this time which was comparable with that on any one of the 24 treated plants. After two days the plants in both concentrations of gas showed noticeable distortions of the leaves. At the end of three days the plants were removed from illuminating gas and photographed (Fig. 6). The effect of illuminating gas on the young leaves of the tulip is very evident from the appearance of the two groups at the right (Fig. 6 B). Many leaves show an arched bending and all of the main leaves show an irregular inward rolling which produces a wavy margin. All of the treated plants are noticeably stunted and have failed to unfold as did those in the control group at the left. Fifteen days after removal from gas these same plants had not unrolled completely, although considerable increase in height had occurred (Fig. 6 C).

Effect of gas on flowering. Young flower buds were usually killed by illuminating gas in concentrations of 1 to 10,000 or higher (Fig. 6). Large flower buds opened during treatment with illuminating gas but the petals of these flowers usually withered prematurely at the tip and along the central vein and dropped sooner than petals on control plants. Withered areas were of a transparency similar in appearance to that described for the narcissus. Flower buds on the plants shown in Figure 6 were killed by either concentration of illuminating gas (1 to 10,000 and 1 to 100).

HYACINTHUS

Retardation of growth. Leaves of the hyacinth were retarded in growth by exposure to illuminating gas in concentrations ranging from 1 to 75 to

1 to 40,000. In certain tests the total elongation of leaves during the first two days of treatment was compared with that for the second two days in gas. The average percentage of retardation for 15 Marconi leaves in 1 to 75 illuminating gas was 62 for the first two days and 74 for the second two days. A duplicate test gave values of 58 for the first two days and 69 for the second two days of treatment. The average difference for the retardation during the first two and the second two days is 12. The average percentages of retardation for two tests with Bismark leaves were 47 and 67 for the first two and second two days respectively. Leaves in these tests were 6 to 12 centimeters in height at the time of treatment.

Flower stalks of the Bismark and Marconi plants were retarded by all concentrations of illuminating gas ranging from 1 to 75 to 1 to 40,000.

Modified leaf growth. Young leaves on some of the treated Bismark plants bent near the tip when exposed to high concentrations of illuminating gas. The response was most pronounced on slender leaves. This type of curvature also occurred on a few of the control plants. Similar curvatures were not observed on any of the Marconi or L'Innocence plants.

Effect of gas on flowering. The degree of injury to hyacinth flower buds depended upon the age of the buds at the time of treatment. Flower buds were most sensitive to illuminating gas when sufficiently developed to show the color characteristic for the variety, but even in this stage of development a concentration of 1 to 75 did not kill the buds. Abnormal flowering was characterized by delayed opening, small flowers, the failure to develop normal color, premature withering, the early appearance of water-soaked areas on the corolla, the failure of petals to open fully, the distortion of petal tips, or the inward (upward) rolling of the edges of petals. The most consistent responses were the failure of tips of petals to turn from green to the variety color and the inability of the flowers to open fully.

Young flower buds entirely green in color were more resistant than leaves to concentrations of gas higher than 1 to 75. In these tests pure illuminating gas was allowed to flow for 10 to 20 seconds into bell jars containing the plants. The bell jars were then sealed and left for six days. The tips of most leaves were killed, but the young flower buds continued to develop after removal from the gas and finally produced flowers on a stunted flower stalk.

DISCUSSION

Retardation of growth. The stems of lilies and the leaves and flower stalks of the narcissus, tulip, and hyacinth were retarded in growth during exposure to illuminating gas. All varieties of the four genera used were retarded by relatively low concentrations of gas. The leaves and flower stalks of narcissus were less retarded by low concentrations (1 to 20,000 or 1 to 40,000) than they were in concentrations two to four times as strong. Within the same limited range of concentration the other genera did not

show a similar correlation between the amount of retardation and the concentration of gas used. Sometimes a concentration of 1 to 20,000 would produce the same amount of retardation as 1 to 100 (Table II). Generally, however, a concentration of 1 to 100 produced a greater retardation than a much weaker concentration such as 1 to 10,000.

One of the most striking characteristics of illuminating gas (or of ethylene) is that it retards growth over an extremely wide range of concentrations and for relatively long periods of exposure without killing the plants. In these experiments the concentrations of illuminating gas ranged from 1 to 75 to 1 to 40,000 and the time of exposure from one to seven days. Death or abscission of the leaves did not occur, and the effect on the rate of growth was not greatly different at the lowest concentration from that 400 to 500 times as strong. Many chemicals and certainly most toxic gases, such as sulphur dioxide, cause stimulation (if any), retarded growth, and death within narrow limits of concentration (two to ten times).

Although the leaves of narcissus, tulip, and hyacinth were consistently retarded in growth during treatment in high concentrations of illuminating gas, in only a few cases was there a cessation of growth even after three to four days' exposure. On the other hand the stems of one-third of the Harrisii and one-fourth of the Giganteum lilies ceased growth during treatment. The response of these two lilies is, therefore, similar to that shown by the rose (4) in which case 28 per cent of the shoots ceased growing during exposures of three to seven days to illuminating gas. The stunting effect of illuminating gas on Easter lilies reported by Crocker (2) is confirmed by the results of our experiments.

Results with the tulip and hyacinth showed that there was a slightly greater retardation of the growth of leaves during the last two days as compared with the first two days of treatment. The values obtained for the tulip were more convincing than those for the hyacinth, but the individual variations were probably too great to make differences of 12 to 25 per cent significant. The cessation of growth was, therefore, an exceptional occurrence rather than a general occurrence.

Flower stalks of the narcissus, tulip, and hyacinth were retarded the same as leaves during treatment with illuminating gas. High concentrations (1 to 100) usually produced a greater amount of retardation than low concentrations when the flower stalk was in the early stages of development.

Modified leaf growth. There were marked varietal differences in specificity, types, and the degree of the responses induced by illuminating gas. Pronounced responses occurred only on young leaves and the response of any one variety depended particularly upon the activity of growth previous to and at the time of exposure to gas. Temperatures between 75° to 85° F. were usually more favorable than temperatures below 75° F. for the epinastic and inward rolling responses.

Epinasty in the form of an arched downward bending occurred consistently on young leaves of the lily, narcissus, and tulip, but not on leaves of the hyacinth. The leaves of narcissus showed both epinasty and hyponasty since in some cases all leaves bent in the same direction (Fig. 6 D). Had the varieties King Alfred and Campernelle narcissus been exposed to illuminating gas when the leaves were younger than is shown in Figure 3 B and C, it is possible that the leaves would have shown curvatures. William Copeland was the only variety of tulip which was in a rapid state of growth at the time of treatment, and it was the only variety of tulip to show arched bending consistently on the treated plants. The main leaves arising from large hyacinth bulbs did not show an epinastic response to illuminating gas, but the slender leaves from small bulbs frequently showed an arched bending or a right angle bend.

Pronounced types of leaf distortion such as curling, irregular inward rolling, twisting, and swelling were decidedly more specific than the less marked responses such as a slight declination and a right angle or an arched bend. All of the pronounced responses were induced by illuminating gas in concentrations of 1 to 10,000 or higher.

Permanent curling occurred only on the Paper White narcissus (Fig. 2). The curl was circular, of small diameter (usually less than one centimeter), and was usually found on leaves not over eight to ten centimeters in height. In contrast, the loop type of curl on Mrs. Langtry narcissus (Fig. 5 D) was arched at the top and V-shaped at the base, large in diameter (one to three centimeters) and was found on leaves from 8 to 16 centimeters in height. A double bending response was frequently associated with the formation of the loop on Mrs. Langtry leaves. The curling on young leaves of the Giganteum and Rubrum lilies was similar to that on leaves of the Paper White narcissus, but it was not permanent. The curls on Giganteum leaves unrolled during treatment as well as after removal from illuminating gas.

Irregular inward rolling occurred consistently on young actively growing leaves of the William Copeland tulip and was distinctly different from the even marginal rolling characteristic of the varieties Idyll, Inglescombe Yellow, and Nectar when the leaves were in the process of unfolding. Twisting was induced by illuminating gas only on the actively growing young leaves of the Giganteum lily. However, since twisted leaves were observed on many of the control plants of Giganteum and Harrisii lilies, all narcissus varieties, and several varieties of tulips, twisting is not regarded as being due entirely to illuminating gas.

The swelling or inflation of the leaves of Mrs. Langtry narcissus was the most specific of the responses induced by illuminating gas. Inflations appeared on the young leaves of all treated plants of the variety Mrs.

Langtry, but not on any of the other varieties of plants tested. This response was distinctly different in character from any of the others.

Bending and curling responses were not always of the epinastic type. On narcissus and tulip leaves there was both epinasty and hyponasty. The irregular inward rolling response of tulip leaves was due to the alternate occurrence of hyponastic and epinastic curvatures along the same margin. There was some evidence that leaves of these two genera tended to bend in the same direction as any slight declination or curvature already present when the plants were exposed to gas. This was particularly true of tulip leaves as may be seen in Figure 6 by comparing the same leaves before and after treatment. Two distinct types of response were sometimes present on the same leaf, as for example, arched bending and inward rolling (tulip, Fig. 6), epinasty and hyponasty (Mrs. Langtry narcissus, Fig. 5 D), and swelling and arched bending (Mrs. Langtry narcissus). In a few cases swelling, epinasty, and hyponasty appeared on the same leaf (Mrs. Langtry narcissus). Some of the tulip leaves on the treated plants in Figure 6 B show outward rolling (epinasty), inward rolling (hyponasty), and either or both arched bending (epinasty at right angles to that shown by outward rolling), and twisting.

As previously mentioned, illuminating gas caused approximately the same amount of retardation of the growth of leaves when used in concentrations over a range of 1 to 75 to 1 to 40,000. Thus the highest concentration was 533 times the lowest. The same range of concentrations produced a modified leaf growth, although the pronounced types of distortion such as curling, swelling, and irregular inward rolling usually required a concentration of 1 to 10,000 at temperatures of 70° to 85° F. Death or abscission of the leaves did not occur and a normal rate of growth was resumed after the plants were removed from gas even though the distortion remained. So far as the leaves are concerned, the lily, tulip, narcissus, and hyacinth are relatively resistant to illuminating gas. Since leaf responses to illuminating gas occurred only under restricted conditions, these four genera cannot be considered as reliable test plants for detecting the presence of illuminating gas or of ethylene. Even under the most favorable conditions the lily, narcissus, and tulip would not have the sensitivity of the tomato plant in detecting traces of illuminating gas (1 to 50,000 to 1 to 200,000).

The results of Doubt (3, p. 217) were not entirely confirmed by our experiments. In reference to the effect of illuminating gas on tulips and hyacinths it is not clear what she means by "the tips of the younger leaves rolled up." Although on our tulips the irregular inward rolling of the first leaf was usually confined to the upper half, in some cases the entire margin was involved (Fig. 6). A more even rolling occurred along the entire margin of the second leaf. No rolling was obtained on the varieties of hyacinths we used. Doubt obtained rolling with a concentration of 10,000 p.p.m. (1

to 100) but not with 4000 p.p.m. (1 to 250). Our results show marked rolling of the leaves of the William Copeland tulip in 1 to 10,000 (100 p.p.m.) illuminating gas (Fig. 6 B) which is 40 times more dilute than the concentration which Doubt found just failed to cause rolling. This disagreement may be due to differences in the age of the leaves, the activity of growth, or the variety of plant used.

Effect of gas on flowering. The effect of gas on flowering was dependent on the concentration of gas, the variety of plant, and the age of the flower buds at the time of treatment. Young flower buds of the lily and narcissus were killed by relatively low concentrations of illuminating gas (1 to 10,000 or 1 to 20,000). A concentration of 1 to 10,000 or higher killed young buds of the tulip. Hyacinth flower buds were not killed during any stage of development by the highest concentration of illuminating gas used (1 to 75). Thus, of the four genera used, the flower buds of the hyacinth were the most resistant to illuminating gas even though slight injury occurred at the relatively low concentrations of 1 to 10,000 and 1 to 40,000. In contrast to these results Doubt (3) failed to obtain injury in 4000 p.p.m. which is equivalent to 1 to 250 of our illuminating gas. This disagreement may be due to differences in the varieties of hyacinths, although it was not stated which varieties she used.

Mature flower buds of all genera usually opened more quickly during treatment with illuminating gas, but these flowers seldom lasted as long as those on control plants. Open flowers were not affected even by high concentrations during the first 24 to 48 hours, but thereafter they aged more quickly than the controls. However, if the flower buds were in the early stages of growth at the time of treatment, they were injured, killed, or noticeably retarded in development so that they failed to open or opened at a later date than those on control plants. These results are similar to those reported for the rose (4) in which it was shown that mature or nearly mature flower buds opened during treatment in advance of those on the control plants, whereas younger buds were either retarded in development or killed.

The abscission or fall of petals or flower buds before they had become shriveled did not occur as a result of treatment with illuminating gas as in the case of the rose (4). In general, flowering was not so consistently interfered with by relatively low concentrations of illuminating gas as was the elongation of the leaves and flower stalks.

SUMMARY

1. The lily, narcissus, tulip, and hyacinth were exposed during different stages of development to illuminating gas in concentrations ranging from 1 to 75 to 1 to 40,000. The time of exposure varied from one to seven days.

2. The lily, narcissus, tulip, and hyacinth were retarded in growth dur-

ing treatment by all concentrations of illuminating gas used without causing death or abscission of the leaves. Narcissus was the only genus which showed differences in the amount of retardation within relatively narrow limits of concentration (two to four times).

3. Pronounced responses of leaves to illuminating gas such as curling, looping, double bending, irregular inward rolling, and inflation depended particularly upon the age of the leaf, the rate of growth, and the variety of plant. All of these responses were obtained over a wide range of concentration (1 to 75 to 1 to 10,000) without resulting in the death or abscission of the leaves. Although with the exception of inflation these distortions were permanent, the leaves continued to grow after removal from gas. Permanent curling occurred only on young leaves of the Paper White narcissus; looping, double bending, and inflation of young leaves were restricted to Mrs. Langtry narcissus; and irregular inward rolling was obtained consistently only on the young actively growing leaves of the William Copeland tulip. Less marked responses such as a slight declination, a right angle bend, or an arched bend occurred on young leaves of the lily, narcissus, and tulip, but not on leaves of the hyacinth. Mature or nearly mature leaves did not show any of the responses described.

4. The effect of illuminating gas on flowering depended upon the age of the flower bud at the time of treatment, the variety of plant, and the concentration of gas used. Young flower buds of the lily, narcissus, and tulip were killed by illuminating gas in concentrations of 1 to 10,000 or higher. Sometimes weaker concentrations killed the flower buds, particularly of the lilies. Medium-aged flower buds were usually injured but not always killed. High concentrations of gas did not kill the flower buds of hyacinth, but the buds were injured if they were partly colored at the time of treatment.

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ETHYLENE-INDUCED EPINASTY OF LEAVES AND THE RELATION OF GRAVITY TO IT

WILLIAM CROCKER, P. W. ZIMMERMAN, AND A. E. HITCHCOCK

INTRODUCTION

It has been known for some time that petioles of leaves of many, but not all, plants grow more rapidly on the upper side, or show chemo-epinasty, in an atmosphere bearing ethylene, artificial illuminating gas, or tobacco smoke. Molisch (15) sealed plants with one to three puffs of cigar or cigarette smoke in bell jars of four to seven liters capacity. The following showed "chemotactic" response of the leaves: *Boehmeria utilis*, *Splitgerbera biloba*, *Boehmeria polystachya*, *Impatiens parviflora*, *I. sultani*, and *Parietaria officinalis*. The first two showed very marked response and the others evident though somewhat weaker response. The horizontal position of the petioles was reached after 24 to 48 hours of exposure to the smoke. After this the petioles continued to grow more rapidly on the upper side until they reached a vertical position. In *Boehmeria utilis* the response continued until the petioles became spiral. The petioles of *Tolmiea menziesii* showed no response under similar conditions. The petioles of the first two plants mentioned above gave similar responses in "laboratory air" and in air containing traces of artificial illuminating gas. Molisch did not determine the constituent or constituents of the several gases that induced this response.

Harvey (10) discovered that leaves of castor bean (*Ricinus communis*) often showed epinasty in the laboratory. He showed that this response was due to traces of ethylene in the air coming from slight leakage of illuminating gas. By experiment he showed that 1 part of ethylene in 10,000,000 of air produced measurable epinastic response in the leaves. He recommended this plant as a reliable and very delicate test plant for ethylene in the air. Doubt (7) found that the leaves of *Salvia splendens*, *Ricinus communis*, and *Datura stramonium* gave epinastic response to 1 part of ethylene to 10,000,000 of air while *Lycopersicum esculentum*, *Coleus* sp., and *Hibiscus rosa-sinensis* required 2 p.p.m. and *Acalypha tricolor* and *Begonia luminosa* required very much higher concentrations for the same response. No epinastic response was observed in the leaves of *Populus deltoides*, *Pelargonium zonale*, and *Caladium esculentum* for any concentration of ethylene or artificial illuminating gas used. Doubt (7, p. 212) stated, "The bending may be near the blade or bud, as in Calla lily leaf and flower; all along the petiole, as in most young leaves; or very near the stem as in most older petioles." Carbon monoxide, according to Doubt, did not produce epinastic response even in the plants most sensitive to ethylene but the highest concentration tried was 1 part in 20,000 of air.

Crocker (6) made use of the epinastic response of the leaves of vigorously growing tomato plants as a means of detecting the presence of traces of artificial illuminating gas in greenhouses where gas injury was suspected. He found the tomato plant to be quick responding and sufficiently sensitive to detect any concentration of gas that would injure the more sensitive plants grown in greenhouses. Zimmerman, Crocker, and Hitchcock (23) mentioned leaves of the following plants as showing epinastic response to artificial illuminating gas in concentrations ranging from 1 part to 100 to 1 part to 50,000 of air: tomato, potato, ageratum, buckwheat, sunflower, castor bean, fuchsia, *Nicandra physalodes*. The tomato responded quickest showing a measurable bending down of the leaf within three hours in 1 part of gas to 10,000 of air. In young tomato leaves the bending extended throughout the entire length of the petiole but in old leaves it was near the base of the petiole. In fuchsia leaves the curvature occurred all along the petiole and blade of the leaf. Temperature modified the rate and amount of the response. The response was rapid and marked at 15° C. or above, less so at 13° C., and there was little, if any, bending at 5° to 10° C. The same authors (25) found that the youngest leaves of the rose showed slight epinastic response to high concentrations of ethylene (1 part to 25,000 of air). This was mainly shown by a sharp bending downward of the leaflets at the base and a ventral inrolling of the edges of the leaflets. It required some experience to distinguish this gas-induced position of the young leaflets from their normal opening position.

Neljubow (16) stated that a number of workers had observed that epicotyls of the pea (*Pisum sativum*) and some other legumes developed in a peculiar way in laboratory air. In the greenhouse they elongated rapidly, were slim, and grew vertically, while in laboratory air they elongated slowly, showed much greater thickness and grew horizontally. He found that this modified growth habit was not due to the dryness of laboratory air nor to sulphur gases, but was due to traces of artificial illuminating gas in the air. Ethylene and acetylene proved especially effective. One part of ethylene in 3,000,000 of air induced the response in only a portion of the epicotyls, but two to four times this concentration gave it in all of them. In a later paper, Neljubow (17) reported results of many experiments on the geo-equilibrium position of the epicotyl of the pea in an atmosphere bearing 2.5 p.p.m. of ethylene. He started with seedlings that had been grown in pure air until they were 5 to 8 cm. high. When such seedlings were placed upright in the ethylene-air mixture they gradually assumed the horizontal position at the tip and continued to grow horizontally. If they were placed in a horizontal position they continued to grow horizontally. If they were placed in an oblique position either above or below the horizontal they assumed the horizontal position. Seedlings grown from the seed stage in the ethylene-air mixture and kept constantly on a rotating hori-

zontal clinostat grew straight and at right angles to the surface of the soil in which they grew. Neljubow concluded that the geo-equilibrium position of the pea epicotyl in such an atmosphere was horizontal or the organ was diageotropic. He also concluded that when the epicotyl was displaced from its equilibrium position in gas the *direction* of bending was always determined by the exposure to gravity and not by the structure of the organ as previous workers had thought. The epicotyl acted physiologically as a radial organ. Neljubow published figures of the gas-exposed and pure-air epicotyls in his first article but not in the second. An examination of these figures shows that most of the epicotyls assumed the declined position by bending in the direction of the closed side of the hook at the tip of the epicotyl. This raises the question whether the *direction* of the bending was not autonomic or internally determined by the structure of the organ as previous workers thought. Undoubtedly the final equilibrium was determined by gravity. An examination of these figures also leads one to question whether Neljubow was not dealing with a "klinogeotropic" (assuming a position other than horizontal or vertical) rather than a diageotropic response according to Pfeffer's definition (18, v. **3**: 155), for most of the epicotyls grown in the impure air were in an oblique rather than a horizontal position.

In the main, Singer (19) confirmed Neljubow's results for the pea and lentil epicotyls and investigated the reason for the like response of the potato sprouts grown in laboratory air. Vöchting had explained the latter as a hydrotropic response, but Singer found that it was caused by traces of artificial illuminating gas in the laboratory air and was quite independent of humidity or differential humidity on the various flanks of the potato sprouts. Potato sprouts had their growth modified in extremely low concentrations of gas. Two parts of illuminating gas to 100,000 parts of air practically stopped elongation and caused a knob-like enlargement near the tip of potato stems. One-half or one-fourth of this concentration permitted considerable elongation and caused horizontal nutation. Singer's work indicated that the potato sprout was even more sensitive to ethylene than the pea epicotyl. An examination of his figures shows that the potato sprouts in assuming the declined position in almost all cases bent in the direction of the closed side of the crooked tip. The direction of bending was apparently determined by the structure of the tip of the stem but the equilibrium position by gravity.

Knight and Crocker (12) and Knight, Rose, and Crocker (13) reported on the effect of several gases on the development of etiolated epicotyls of the sweet pea. The epicotyls were grown in air in darkness to a height of 3 cm. before they were placed in the air-gas mixtures and left for three days. They found that ethylene, acetylene, propylene, carbon monoxide, cigar and cigarette smoke, and artificial illuminating gas produced the "triple

response": inhibition of elongation, increasing growth in diameter, and declination or horizontal nutation of the region growing while in the gas. Extremely low concentrations of ethylene produced these growth modifications. One part of ethylene in 10,000,000 of air produced marked inhibition of growth but no swelling or declination from the vertical. Twice this concentration inhibited elongation still more and caused several degrees declination from the vertical but no swelling. Four parts of ethylene to 10,000,000 of air inhibited elongation still more, caused swelling of the growing region, and a declination approaching the horizontal. Still higher concentrations inhibited elongation so completely that the seedling stood upright with a swollen knob near the tip due to increased growth in diameter. Calculated on the basis of the minimum concentrations of the various gases necessary to produce these responses to a like degree, ethylene was 25 to 50 times as effective as water gas, 1000 times as effective as acetylene, 2500 times as effective as propylene, and 25,000 times as effective as carbon monoxide. Their work showed that ethylene was the highly effective constituent in cigar and cigarette smoke and in artificial illuminating gas in producing this modified growth and geotropic response. Turpentine, benzene, ethyl ether, chloroform, and benzine in suitable partial vapor pressures in the air reduced the rate of elongation and caused swelling in the growing tip of the sweet pea but did not, regardless of concentrations used, produce declination or horizontal nutation. Hydrogen sulphide, carbon bisulphide, and sulphur dioxide in proper concentrations inhibited rate of elongation, but did not, in any concentration, cause swelling or modified geotropic response. It will be noted that all the gases that produced the "triple response" were carbon-containing gases with unsaturated bonds.

Knight and Crocker (12) and Crocker (5) published a number of figures of sweet pea seedlings grown for three days in the various gases producing the "triple response." An examination of these figures led to some interesting conclusions. So far as can be determined all tip portions of the seedlings in assuming the declined position bent in the direction of the closed side of the hook at the tip or had the open side of the hook facing upward. The *direction* of the bending was autonomic or determined by the differential structure of the tip contrary to Neljubow's conclusion and in accordance with the views of older workers. The declined top portions of the seedlings growing while in the optimum concentration of gas for declination never reached the horizontal position but were declined at most only 70° to 85° from the upright position. The equilibrium position was a "klinogeotropic" position rather than a diageotropic position. These authors were evidently not justified in using Neljubow's term, "diageotropism," to describe the position of these seedlings. From the discussion above one may conclude that the *direction* of bending of legume epicotyls and potato sprouts when

placed in air containing traces of ethylene and certain other gases was autonomic and determined by the non-radial character of the tip of the organ, that the final declination represented an equilibrium position with gravity, and that this equilibrium position was a "klinogeotropic" position and not a diageotropic position.

Wallace (20, p. 395) found an interesting relation between the location of ethylene-induced intumescences on apple twigs and their orientation to gravity. Twigs in the upright position formed intumescences near the morphologically apical end and inverted twigs near the morphologically basal end.

There are three factors (2) that have been considered of importance in determining the position of leaves. The gravity stimulus has been considered important for all leaves and light significant for some leaves and of little or no significance for others. Autonomic epinasty, that is, the internally determined stimulus due to which leaves grow faster on the upper side when relieved of the restraining influence of the gravity stimulus, has also been considered of significance in determining the position of leaves. Kneip (2) rotated plants of *Lophospermum* on an intermittent clinostat in such a way that the two flanks of the petioles had successively like exposures to gravity for equal intervals thus equalizing the gravity stimulus on the several faces of the petioles. Leaves thus treated grew more rapidly on the upper side or showed autonomic epinasty. For any individual leaf, however, in its natural position it is impossible to say to what extent that position is determined by the gravity stimulus and to what extent by autonomic epinasty.

This paper extends the knowledge of chemo-epinasty of leaves in several directions and attempts to show the part that the gravity stimulus plays in it.

RESULTS

1. *Frequency of occurrence and variation in response.* In the extensive studies made in this laboratory on the effects of artificial illuminating gas and ethylene on plants, 202 different species and varieties of plants have been tested for ethylene-induced epinasty of the leaves. This is exclusive of the study of the effect of ethylene on several species belonging to the four genera *Hyacinthus*, *Lilium*, *Narcissus*, and *Tulipa*, the results of which are published elsewhere in this Number of this Journal. For every sort of plant at least 10 p.p.m. of ethylene were used and for most sorts much higher concentrations. Ten p.p.m. is 100 times the concentration necessary to cause response in the more sensitive plants.

The following 72 showed marked epinasty of the leaves: *Ageratum houstonianum* Mill.; *Amaranthus retroflexus* L.; *Anethum graveolens* L.; *Asclepias curassavica* L.; *Browallia speciosa* Hook.; *B. viscosa* HBK.; *Bryophyllum pinnatum* Kurz.; *Capsicum frutescens* L. (13 out of 31 varie-

ties tested); *Chenopodium album* L.; *C. ambrosioides* L. var. *anthelminticum* (L.) Gray; *Cosmos bipinnatus* Cav.; *C. sulphureus* Cav.; *Datura suaveolens* Humb. & Bonpl.; *Euphorbia heterophylla* L.; *Fagopyrum esculentum* Moench; *Fuchsia hybrida* Voss; *Galinsoga parviflora* Cav.; *Gomphrena globosa* L.; *Gossypium hirsutum* L.; *Hedera helix* L.; *Helianthus debilis* Nutt.; *Impatiens balsamina* L.; *Lantana lilacina* Desf.; *Lathyrus odoratus* L.; *Linum grandiflorum* L.; *Lycopersicon esculentum* Mill. (8 varieties); *L. pimpinellifolium* Mill.; *Malva rotundifolia* L.; *Medicago sativa* L.; *Melilotus alba* Desr.; *Nepeta cataria* L.; *Nicandra physalodes* (L.) Pers.; *Nigella damascena* L.; *Petroselinum hortense* Hoffm.; *Piqueria trinervia* Cav.; *Pisum sativum* L.; *Radicula nasturtium-aquaticum* Britten & Rendle; *Ricinus communis* L.; *Salvia splendens* Ker.; *Solanum auriculatum* Ait.; *S. aviculare* Forst.; *S. bonariense* L.; *S. dulcamara* L.; *S. nigrum* L.; *S. nodiflorum* Desv.; *S. tuberosum* L.; *Tagetes erecta* L.; *T. patula* L.; *Tropaeolum majus* L.; *Vigna catjang* Walp.; *Viola tricolor* L.; *Zinnia elegans* Jacq.; and *Z. haageana* Regel.

The following 17 showed slight epinasty of the leaves: *Coleus blumei* Benth.; *Euphorbia pulcherrima* Willd.; *Heliotropium peruvianum* L.; *Nicotiana glutinosa* L.; *Physalis viscosa* L.; *Rosa* (hybrid tea, 6 varieties)[1]; *R. canina* L.[1]; *R. hugonis* Hemsl.[1]; *Solanum flavum* Kit.; *S. melongena* L. var. *esculentum* Nees.; *S. sanitwongsei*; and *Wisteria sinensis* Sweet.

The following 113 showed no epinasty of the leaves: *Acer palmatum* Thunb.; *Antirrhinum majus* L.; *Asparagus sprengeri* Regel; *Avena sativa* L.; *Begonia coccinea* Hook.; *B. rex* Putz.; *Brassica oleracea* L. var. *capitata* L.; *Calendula officinalis* L.; *Callistephus chinensis* Nees.; *Cannabis sativa* L.; *Capsicum frutescens* L. (18 out of 31 varieties tested); *Carica papaya* L.; *Caryocar villosum* Pers.; *Cattleya luddemanniana* Reichb.; *Centaurea cyanus* L.; *Cheiranthus cheiri* L.; *Clarkia elegans* Dougl.; *Coix lacryma-jobi* L.; *Commelina communis* L.; *Coreopsis tinctoria* Nutt.; *Cucumis melo* L.; *C. sativus* L.; *Cymbidium insigne* Rolfe; *Cynara scolymus* L.; *Dianthus barbatus* L.; *D. chinensis* L. var. *heddewigii* Regel; *Digitalis purpurea* L.; *Eschscholzia californica* Cham.; *Euphorbia marginata* Pursh.; *Freesia hybrida* Hort.; *Gardenia jasminoides* Ellis; *Gladiolus* sp.; *Glycine max* Merr.; *Gypsophila paniculata* L.; *Hordeum vulgare* L.; *Hydrangea opuloides* Koch; *Hyoscyamus albus* L.; *Lactuca sativa* L.; *Lepidium sativum* L.; *Linaria vulgaris* Hill; *Lobularia maritima* (L.) Desv.; *Majorana hortensis* Moench; *Mathiola incana* R. Br. var. *annua* Voss; *Mimosa pudica* L.; *Mirabilis jalapa* L.; *Myosotis scorpioides* L.; *Nephrolepis exaltata* Schott. var. *bostoniensis* Davenport; *Nicotiana acuminata* Hook.; *N. alata* Link & Otto; *N. clevelandi* A. Gray; *N. langsdorfi* Schrank; *N. longiflora* Cav.; *N. multivalvis* Lindl.; *N. palmeri* A. Gray; *N. paniculata* L.; *N. plumbaginifolia* Willd.; *N. quadrivalvis* Pursh.; *N. rusbyii*; *N. rustica* L.; *N. sanderae* San-

[1] Only leaflets of youngest leaf.

der; *N. suaveolens* Lehm.; *N. tabacum* L. var. Burley; *N. tabacum chinensis* Fisch.; *N. tabacum* L. var. Connecticut Seed Leaf; *N. tabacum* L. var. *gigantea; N. tabacum* L. var. Greene's Wildfire Resistant Orinoco; *N. tabacum* L. var. Turkish; *N. trigonophylla* Dun.; *Oenothera lamarckiana* Ser.; *Oncidium splendidum* A. Rich.; *Papaver rhoeas* L. var. Shirley; *Pelargonium graveolens* L'Her.; *P. hortorum* Bailey; *Persea americana* Mill.; *Petunia hybrida* Vilm.; *Phaseolus vulgaris* L.; *Phlox drummondii* Hook.; *Physalis francheti* Masters; *Polygonum pennsylvanicum* L.; *Primula malacoides* Franch.; *P. obconica* Hance var. *gigantea; Raphanus sativus* L.; *Reseda odorata* L.; *Rosa chinensis* Jacq. var. *manetti* Dipp.; *Salpiglossis sinuata* Ruiz & Pav. var. *superbissima; Salvia officinalis* L.; *Secale cereale* L.; *Silene pendula* L.; *Solanum indicum* L.; *S. pseudocapsicum* L.; *Syringa vulgaris* L.; *Taraxacum officinale* Weber; *Trachymene caerulea* R. Graham; *Trifolium pratense* L.; *Zea mays* L. var. *rugosa* Bonaf.; and *Zebrina pendula* Schnizl.

About 36 per cent of the 202 species and varieties tested showed marked epinasty of the leaves, 8 per cent slight, and 56 per cent none.

Doubt (7) found that young leaves showing ethylene-induced epinasty curved along the whole length of the petioles while in older leaves the petioles bent near the stem of the plant. She found some exceptions to this. Molisch (15) stated that the bending of the petioles generally continued until the petioles were parallel to the stems of the plant. He found one exception to this. In *Boehmeria utilis* the epinastic response continued until the petiole became spiral. We also found some exceptional types of ethylene-induced epinasty of leaves. Some of these are shown in Figure 1. All the plants shown in this figure were sealed in Wardian cases under the conditions mentioned in the description of the figure. In the case of fuchsia ethylene caused the petioles to decline somewhat but most of them did not reach the horizontal position. The main epinastic response was in the blade of the leaf and consisted of a curvature which extended the whole length of the blade. This threw the tips of the blades to or beyond the vertical position. While a very high concentration of ethylene was used in this case, fuchsia showed a similar response to 2 p.p.m. of ethylene in air with 20 hours' exposure. It is no doubt one of the more sensitive plants to ethylene. In the case of buckwheat and sunflower plants, ethylene produced extreme epinasty and torsion of some of the petioles. In the buckwheat one such case appears on the right-hand plant about one-third of the way from the top, and a more extreme case on the left-hand plant about one-fourth of the way from the top. A careful examination of the buckwheat shows a number of other petioles with extreme epinasty and torsion. On the sunflower a petiole near the top of the plant shows extreme distortion, and the petiole next below it considerable distortion. For these two plants a relatively high concentration of gas was used, 1 part of Yonkers water gas to 10,000 of air.

FIGURE 1. *Ethylene-induced epinasty of leaves.* All plants sealed in Wardian cases for the periods and in the concentrations of gases mentioned below and photographed immediately after removal from the cases: 1. Fuchsia, check; 2. Fuschsia in 500 p.p.m. of ethylene for 24 hours; 3. Buckwheat, 1 part to 10,000 of Yonkers gas, equivalent to 3 p.p.m. of ethylene, for 72 hours; 4. Sunflower, 1 part to 10,000 of Yonkers gas, 48 hours; 5. Paper White narcissus, 1 part to 10,000 of Yonkers gas, 96 hours.

This gas had an ethylene content of 3.4 per cent, so the ethylene content in the case was about 3 p.p.m. In other experiments these plants showed epinastic response of the leaves in 0.05 p.p.m. of ethylene. They proved as sensitive as any plant tested. As the figure shows, the epinasty of the Paper White narcissus leaves was limited to the tip of the younger leaves. In the youngest leaves flat or skew spirals were formed. The next older leaves showed evident epinastic bending of the tip region of the leaves. In this experiment gas giving the equivalent of 3 p.p.pm. of ethylene was used. Other experiments showed that one-half this concentration did not produce epinasty, while any concentration greater than 3 p.p.m. equivalent of ethylene up to the highest tested which was 2000 p.p.m. equivalent of ethylene did produce the epinasty. Paper White narcissus proved to be one of the less sensitive plants to ethylene.

The results reported in this section show that of the 202 different species and varieties of plants tested for ethylene-induced epinasty of the petioles, 89 showed it and 113 did not. The results also show that plants vary considerably in the type of epinasty shown and greatly in the concentration of ethylene required to produce the response.

2. *Growth of petioles during ethylene-induced epinasty and during recovery from it.* In studying the change in length of the upper and lower faces of petioles during epinastic response in ethylene-air mixtures and recovery from it in air, tomato plants were used and only the petioles of intermediate age were measured. The younger three or four leaves on the plant proved unfavorable because growth occurred throughout the length of the petiole making measurements of many spaces on each face necessary with very slight increments in each; also the normal growth of the petioles masked the induced growth. The older leaves were not used because, while they responded fully to ethylene, they showed only a slight degree of recovery when placed in air again. The use of the intermediately aged petioles avoided to a great degree all these difficulties; the growth was limited to the region of the petiole near the stem making the increments easily measurable and the recovery, while not complete, was easily determined.

By use of a multiple marker, cross lines of India ink three millimeters apart were made on the upper and lower sides of the petioles beginning at the stem and extending outward several spaces beyond the region showing the growth response to the gas mixture. The numbering of the spaces began with the one nearest the stem and extended outward in consecutive order. Figure 2 shows the change in distances between these cross lines on the upper side of a petiole of a plant (right) that had been subjected for 24 hours to 1 part of Yonkers gas to 10,000 of air in a sealed Wardian case and petioles of a similar plant (left) that had been similarly treated without gas. The plants were decapitated just prior to photographing. On the petiole of

TABLE I

GROWTH OF TOMATO PETIOLES DURING RESPONSE TO ETHYLENE AND RECOVERY IN AIR AS SHOWN BY CHANGE IN LENGTH OF THE 3-MM. SPACES MARKED ON THE UPPER AND LOWER FACES OF THE PETIOLES

Petiole No.	Space No.*	Measurements during intermittent exposures and recoveries								Changes in angle formed by upper side of petiole with stem during intermittent exposures and recoveries				
		After 24 hrs. exposure		After 48 hrs. recovery		After 24 hrs. exposure		After 48 hrs. recovery		Before exposure	After 24 hrs. exposure	After 48 hrs. recovery	After 24 hrs. exposure	After 48 hrs. recovery
		Upper side	Lower side	Upper side	Lower side	Upper side	Lower side	Upper side	Lower side					
1	1	5.0	3.0	5.2	5.0	6.5	4.6	6.9	5.9	45°	110°	70°	130°	90°
	2	5.0	3.0	5.0	4.3	5.1	4.3	5.2	6.5					
	3	3.0	3.0	3.6	3.2	3.6	3.6	3.6	3.6					
2	1	4.6	3.0	5.2	4.5	6.5	4.5	7.1	5.0	60	130	115	130	115
	2	4.0	3.0	4.3	4.0	4.5	3.7	4.7	5.0					
	3	3.0	3.0	3.0	3.2	3.1	3.0	3.1	3.0					
3	1	5.2	3.0	5.3	4.7	7.5	4.5	7.7	5.8	70	130	110	135	110
	2	3.5	3.0	4.3	4.0	4.8	3.9	4.9	5.5					
	3	3.4	3.0	3.7	3.2	3.8	3.8	3.8	3.8					
4	1	4.5	3.0	5.3	3.6	6.3	3.5	6.7	4.5	60	140	100	130	115
	2	4.1	3.0	4.5	4.2	4.9	4.4	5.8	4.7					
	3	3.5	3.0	3.7	3.7	3.7	3.7	3.7	3.7					

TABLE I (*Continued*)

GROWTH OF TOMATO PETIOLES DURING RESPONSE TO ETHYLENE AND RECOVERY IN AIR AS SHOWN BY CHANGE IN LENGTH OF THE 3-MM. SPACES MARKED ON THE UPPER AND LOWER FACES OF THE PETIOLES

5	1	4.0	3.0	4.6	4.0	5.0	3.5	5.2	4.4	40	130	70	150	110
	2	4.0	3.0	4.6	4.3	5.0	4.2	5.2	4.8					
	3	3.3	3.0	3.5	3.4	3.9	3.4	4.1	3.4					
6	1	4.6	3.0	4.8	4.3	6.8	4.0	7.0	6.0	55	140	100	125	100
	2	3.4	3.0	4.7	4.0	4.9	4.2	5.4	4.8					
	3	3.3	3.0	3.5	3.6	3.5	3.6	3.5	3.6					
7	1	4.4	3.0	4.9	4.2	6.0	3.7	6.2	4.7	50	145	90	135	110
	2	4.4	3.0	5.0	4.8	6.0	4.6	6.0	5.3					
	3	3.3	3.0	3.3	3.2	3.3	3.2	3.3	3.2					
8	1	5.0	3.0	5.2	5.0	6.4	4.1	7.0	5.5	40	130	80	130	100
	2	4.0	3.0	4.4	5.0	4.9	5.0	5.2	5.3					
	3	3.3	3.0	3.6	3.7	3.6	3.7	3.6	3.7					
9	1	5.0	3.0	5.2	4.0	5.8	4.0	6.0	5.5	45	145	80	145	110
	2	3.7	3.0	3.9	5.5	4.1	4.3	4.5	5.5					
	3	—	—	—	—	—	—	—	—					
10	1	4.5	3.0	5.2	5.3	6.8	5.0	7.4	7.1	50	125	75	125	100
	2	3.7	3.0	4.2	4.7	5.3	4.5	6.0	5.3					
	3	—	—	—	—	—	—	—	—					
Average	1	4.7	3.0	5.1	4.5	6.4	4.1	6.7	5.4	51	132	89	133	106
	2	4.0	3.0	4.5	4.4	4.9	4.3	5.3	5.2					
	3	3.3	3.0	3.5	3.4	3.5	3.5	3.6	3.5					

* The three-millimeter spaces on the petioles were numbered consecutively from the stem outward.

the gassed plant it can be seen that the space nearest the stem has nearly doubled in length while the second space has lengthened noticeably and the other spaces show little or no elongation. The petioles on the check plants show no elongation of any of the spaces. The epinasty in gas was brought about by an *ethylene-induced* growth of the upper face of the base of the petiole.

Table I shows the measurements of spaces one, two, and three on the upper and lower sides of ten petioles after each of two 24-hour exposures to

FIGURE 2. Tomato plants showing change in length of upper faces of petioles when sealed in Wardian cases for 24 hours: A. In air; B. In 1 part of Yonkers gas to 10,000 of air.

1 part of Yonkers gas to 10,000 of air and after the 48 hours, recoveries from each. At the bottom of the table the averages of the measurements of these spaces for the ten petioles are given. Figure 3 (top) shows the average angles that these ten petioles formed with the stems at the beginning and after each response and recovery. Three sets of curves at the bottom of this figure show the average length of the first, second, and third 3-mm. space on the upper and lower sides of the ten petioles after each response and recovery. The response involved only the petioles and was independent of the rest of the plant, for it occurs when the leaf was removed and kept moist in its normal orientation to gravity.

From the figure, table, and curves, several conclusions are evident. Both the response and recovery were brought about by growth. This

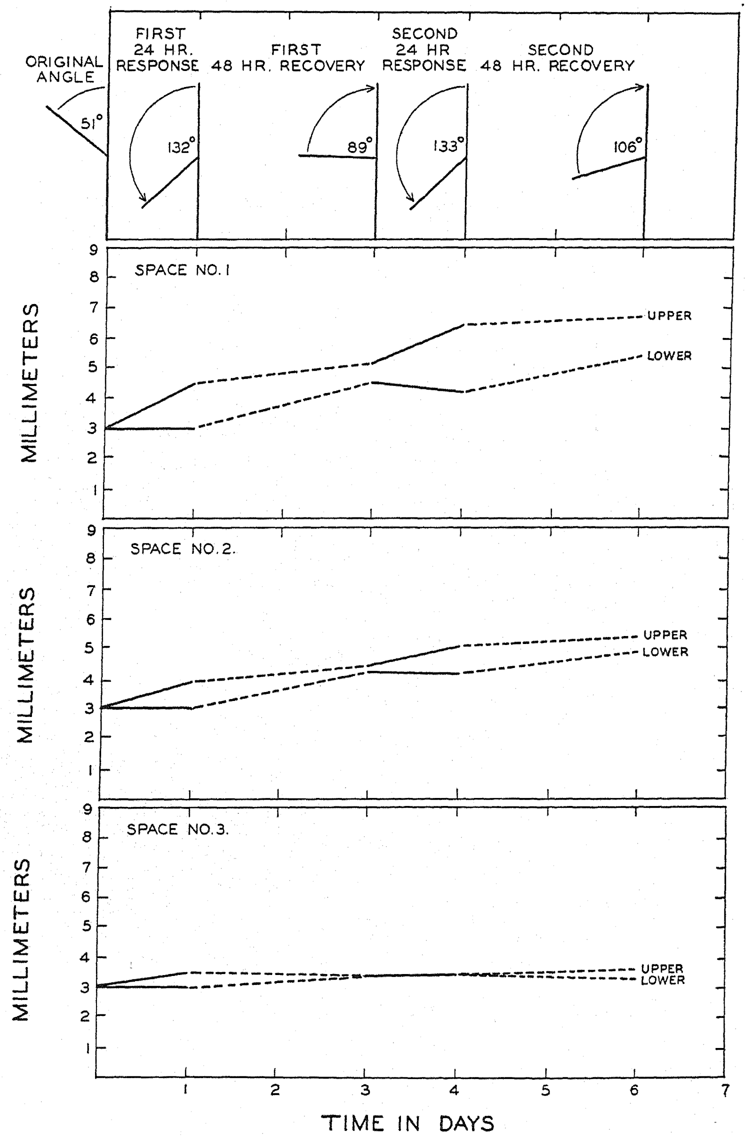

FIGURE 3. Graphic illustration of the average epinastic response of 10 young tomato petioles during intermittent exposures to ethylene and during recovery in air. The uppermost graph shows how the angle formed by the petiole and the stem changed during treatment and recovery. At the beginning the average of the angles was 51°. The other graphs show the change in distance between lines stamped 3 mm. apart on the upper and lower sides of the petiole. The 3-mm. space No. 1 was that nearest the stem; No. 2 the second 3-mm. space; and No. 3 the third 3-mm. space. Data used in making this graph are recorded in Table I.

growth was induced in a region of the petiole that had ceased to grow. The growth that produced the response was induced by ethylene acting, as will be seen later, in conjunction with gravity when the petioles were in their normal orientation to the earth. The growth that led to recovery may have been due to either geotropism or autotropism or both. Considerable stress was produced in tissues of the petiole as was shown by the shortening or compression of the first space on the lower side of the petiole during the second response (see Section 6 of Results). This was also made evident by the great increase in stiffness or tension of petioles of all plants that had responded to gas in contrast with the petioles of check plants growing in air.

3. *Motion pictures of the response and recovery.* Motion pictures were made of tomato and sunflower plants in air and in air containing 2 p.p.m. of ethylene, for the purpose of getting a record of all movements and the order in which they occurred. The pictures were taken in late July and early August, 1931. Two similar plants grown in a greenhouse to the height of 10 to 12 inches were sealed in adjacent rectangular glass cases, one containing air and the other the ethylene-air mixture, and photographed 96 times per hour for 24 hours. The cases were then opened, the plants removed, and the cases aired out. After this the plants were resealed in the cases and exposures made for 24 hours to show the recovery of the gassed plant. The experiments were conducted in darkness except for the temporary illumination during each photographic exposure. After the films were completed they were run at a speed that increased the actual rate of movement 600 times.

In the check tomato plant the younger leaves on the elongating portion of the stem were in continuous nutation. Besides this continuous nutation these leaves showed a night movement which consisted of a ventral inrolling of the tips of the leaves and leaflets. This continued until the upper leaves formed a globular mass with about one-third the diameter of the day spread of the top of the plant. The experiment was begun at 2:34 p.m. and the inrolling started at 5:00 p.m. At about 7:00 a.m. the leaves began to expand again and remained in the extended position until about 7:00 or 8:00 p.m. when they again assumed the night position but the inrolling was not so great the second night. The inrolling the first night was no doubt partly due to the prior change in the light condition, that is, the transfer from the greenhouse to the photographic room. This accounts for its occurring so early in the evening. The inrolling the second night could not have been due to a previous change in light condition but must have resulted from the retained day and night habit; hence its occurrence at a later hour. The weaker movement the second night was probably partly due to the absence of the regular daily change from light to darkness and partly due to a fall in tonus resulting from lack of photosynthesis or other

light effects. This inrolling of the tips of the leaves and leaflets was not accompanied by any noticeable modification of the angle between the petioles and the stem. Observation of a large number of tomato plants in greenhouses showed that the night movement started at dusk, but the amount of inrolling varied considerably with the variety and with different plants of the same variety, and in most cases was less than the inrolling noted in this picture. It was quite distinct from ethylene-induced epinasty as the following paragraph will show, but a careless observer might mistake it for the latter movement when using the tomato as a test plant for ethylene. The older leaves on the part of the stem that had ceased elongation showed no movements and the upper side of the petioles of these leaves formed an acute angle with the stem.

The movements of the gassed tomato plant must be considered under two heads: the response movements and the recovery movements. In the gassed plant neither nutation nor the night movement of the younger leaves occurred while the plant was in gas. Instead, noticeable epinasty of the petioles began within two to three hours after exposure. The first leaf to show this was the third leaf from the top, followed successively by the leaves below on the growing part of the stem and then by the two tip leaves. Several hours later the older leaves on the non-elongating part of the stem showed epinastic movement. The leaves on the growing part of the stem had completed their epinastic movements within 8 or 10 hours and the leaves on the more mature part of the stem within 15 or 20 hours. Younger leaves curved throughout the length of the petioles while in the older leaves the curvature was limited to the base of the petioles. Two or three hours after the gassed plant was put into ethylene-free air, the recovery from the epinasty began and took place in about the same order and, for the younger leaves, with nearly the same speed as the response. The leaves on the growing part of the stem showed complete recovery to the original position, while the older leaves showed only partial recovery. Even after 24 hours the upper surfaces of the older petioles still formed an obtuse angle with the stems.

The motion picture of the sunflower was started at 10:35 a.m. The tip of the check plant began to circumnutate at about 3:30 p.m. and continued until about 7:00 a.m. There was no movement of the plant during the second day but circumnutation began again at 7:00 or 8:00 p.m. and continued until about 7:00 a.m. From that time until the end of the experiment the plant showed no movement. The early initiation of circumnutation the first day was likely due to the changed light condition. The circumnutation the second night must have been due to a retained night habit.

In the gassed sunflower plant the order and rate of epinastic response of the leaves was very similar to that in the tomato plant, as was the

recovery when the plant was placed in ethylene-free air. During the first night when the plant was in the ethylene-air mixture there was no circumnutation of the top. When the plant was recovering in pure air it showed the night circumnutation as did the check plant.

From the descriptions above it is evident that ethylene acted in two ways: first, as an anaesthetic, or rigor producer, stopping the growth movements that occurred in air and, second, as a stimulant, inducing epinastic growth of the petioles which did not occur in air. Knight and Crocker (12) found for the epicotyl of the sweet pea that the lowest effective concentration of ethylene merely reduced the rate of elongation. With increasing concentrations elongation became less and less until in sufficiently high concentrations it ceased altogether. As the concentrations rose and the elongation rate fell off, at first slight declination from the vertical occurred, then greater declination with increased growth in diameter, and finally at the higher concentrations there was only an increased growth in diameter which formed a knob near the end of the upright epicotyl. The anaesthetic action of ethylene manifested itself by reduction in rate of elongation and the stimulative action by an increased growth in diameter. Harvey (11) showed that the increased growth in diameter involved both hypertrophy and hyperplasia. Many plants have been studied in this laboratory as to the effect of ethylene or illuminating gas upon them (23, 25, and unpublished work). The most general reaction found in these studies was the reduced rate or complete inhibition of elongation. Frequently also ethylene induced growth of dormant organs or tissues such as dormant buds, lenticular, abscission, or cork cambium tissues. In these studies the anaesthetic as well as stimulative action was evident.

4. *Gases and vapors causing epinastic response of leaves.* It has already been mentioned that Knight and Crocker (12) found that carbon monoxide and the three unsaturated hydrocarbons tested (ethylene, acetylene, and propylene) produced "klinogeotropism" in the sweet pea seedling but that none of the many other gases and vapors tried had this effect.

The following gases and vapors, all used in a wide range of concentrations varying from those that had no effect to those that gave severe burning of the foliage, did not cause epinasty of tomato petioles: a saturated derivative of ethylene (ethylene chlorhydrin); members of the paraffin series (methane, ethane, propane, butane, and benzine) all present in illuminating gas and the latter containing several members of the series; benzene ring compounds (benzene, cumene, naphthalene, toluene, and xylene) all constituents of illuminating gas; anaesthetics (chloroform[2] and ethyl ether) not present in illuminating gas; sulphur-bearing gases and

[2] Chloroform caused slight declination of some of the older petioles and torsion and downward curving of the outer ends of the leaves, but did not give uniform epinasty of all petioles as did ethylene.

vapors (carbon bisulphide, hydrogen sulphide, thiophene, and sulphur dioxide) the first three of which are generally present in illuminating gas; nitrogen compounds (ammonia and pyridine) both constituents of illuminating gas and the latter with the nitrogen in the ring; the alcohols (ethyl, propyl, isopropyl, and allyl); the aldehydes (formaldehyde, acetaldehyde, and acrolein); isoprene, acetone, bromine, chlorine, carbon dioxide, and turpentine.

There are several explanations necessary concerning the gases and vapors mentioned above that were not effective in inducing epinasty. First, it was difficult to obtain saturated hydrocarbon gases (methane, ethane, propane, and butane) free from unsaturated hydrocarbons. One sample of ethane obtained and said to contain some ethylene gave a marked epinastic response of tomato petioles when 1 part of it was placed in 100,000 parts of air, indicating the presence of about 3 per cent of ethylene. When this ethane was thoroughly washed with bromine water and the bromine later absorbed by several volumes of water, 1 part of the ethane to 10 of air did not induce epinastic response of tomato petioles. This showed that bromine absorbed the ethylene completely or practically completely, for if any ethylene was left in the ethane it was less than 1 p.p.m. In the early attempts to free the ethane of ethylene by first exposing to bromine vapors followed by absorbing the bromine water with 45 per cent KOH, the resulting gas always induced the epinastic response. The bromine apparently reacted with some of the ethane forming ethyl bromide. The vapor of ethyl bromide reacted with KOH forming ethylene. Propane and butane were freed from unsaturated hydrocarbons in the same manner. The highest concentration of propane and butane tested on the plants was 6 parts per 100 parts of air. In this concentration propane did not injure tomato plants but butane caused some burning of the foliage. Neither produced epinasty of tomato petioles.

Three of the vapors mentioned above as not effective in inducing epinasty have carbon chains with double bonds; acrolein ($CH_2=CH-CHO$); allyl alcohol ($CH_2=CH-CH_2OH$); and isoprene ($CH_2=CH \cdot C[CH_3]=CH_2$). The first two are respectively the corresponding aldehyde and alcohol of propylene, which in a later paragraph will be shown to produce epinasty of tomato petioles in concentrations of 50 p.p.m. of air. Both of these proved rather toxic and neither produced epinasty in any concentration. In the case of allyl alcohol 125 p.p.m. in air killed all the foliage of the tomato in 24 hours. In 50 p.p.m. the plant was not injured, was capable of phototropic response while in the vapor, but the petioles did not show the epinastic response. If allyl alcohol were as effective as propylene in inducing epinasty this concentration should have induced it. Twenty-five p.p.m. of allyl alcohol in air did not injure the plant and did not induce petiole epinasty. Acrolein was more toxic than allyl alcohol. Fifty or 30

p.p.m. of the vapor in the air killed all foliage on the tomato plant within 24 hours. Fifteen p.p.m. neither injured the plants nor induced epinasty but the plant responded phototropically in this concentration of the vapors. The saturated three carbon chain alcohols, propyl, and isopropyl, were considerably less toxic. Neither of these were injurious in 200 p.p.m. of the vapor in air. Isoprene was not toxic to the plant when the vapor constituted 3 per cent of the air. When isoprene constituted 10 per cent of the air the tomato plant was killed in three days. Isoprene did not induce epinasty of the leaves in any concentration. In a later part of this section it will be shown that the effectiveness of members of the olefine series falls off very rapidly as the number of carbon atoms in the chain increases. The long carbon chain may account for the ineffectiveness of isoprene in spite of the two double bonds.

The following gases produced epinasty in tomato petioles: acetylene, butylene, carbon monoxide, ethylene, and propylene. The carbon monoxide used in these experiments was derived from heating oxalic acid with sulphuric acid and scrubbing the resulting gas with sodium hydroxide solution to remove the carbon dioxide. The gas used in the tests was collected after the generation had continued long enough to displace all air from the apparatus. The ethylene, acetylene, propylene, and butylene were especially made for this work by the Union Carbide and Carbon Corporation or its subsidiaries and showed the following composition according to analyses furnished by the companies:

Ethylene		*Acetylene*		*Propylene*		*Butylene*	
Ethylene	97.2%	Acetylene	99.8%	Propylene	97.3%	Butylene	86.6%
Oxygen	0.5%	Air	0.2%	Propane	2.6%	Butane	11.6%
Absorbed by KOH	0.3%			Ethane	0.1%	Pentane	1.2%
Nitrogen	2.0%					Propane	0.6%
						Propylene	0.0%

Table II, third column, shows the minimum number of parts per million by volume of each of these gases in air necessary to produce evident

TABLE II

COMPARATIVE EFFECTIVENESS OF GASES IN PRODUCING EPINASTY IN TOMATO PETIOLES AND DECLINATION IN SWEET PEA SEEDLINGS

Gas	Minimum parts per million needed to produce	
	Declination in sweet pea seedlings, according to Knight and Crocker*	Epinasty in tomato petiole**
Ethylene	0.2	0.1
Acetylene	250.0	50.0
Propylene	1000.0	50.0
Carbon monoxide	5000.0	500.0
Butylene	—	50,000.0

* 3 days' exposure used; ** 2 days' exposure used.

epinasty in tomato petioles. From this table it is evident that if the minimum concentration of ethylene necessary to produce the response is considered as 1, the minimum concentrations of the other gases are: acetylene and propylene 500, carbon monoxide 5000, and butylene 500,000. Acetylene and propylene are 1/500 as effective as ethylene in producing epinasty in the petioles of the tomato; carbon monoxide 1/5000 as effective; and butylene 1/500,000 as effective.

The question naturally arises whether the solubilities of these gases may not account for their differences in effectiveness in producing leaf epinasty. The following shows the number of cc. of each gas dissolving in 100 cc. of water when the partial pressure of the gas is 76 cm. of mercury: at 20°C. (14) 103 cc. of acetylene, 12.2 cc. of ethylene, 2.32 cc. of carbon monoxide; and at 18.3°C. 21.64 cc. of propylene. It is evident that there is no relation between solubility in water and effectiveness in inducing epinasty. One is, of course, unable to say how much these solubilities will vary when protoplasm instead of water is used as the solvent. Since water is the dispersal phase in protoplasm, one would expect similar solubilities in protoplasm. It has already been shown (24) that ethylene readily permeates all parts of the plant, probably through the intercellular system, and recent unpublished determinations show the same for carbon monoxide, acetylene, and propylene. It is far more likely that the relative effectiveness of the several gases is determined by their ability to react with certain constituents of the protoplasm.

There are three isomers of butylene but there was no way of knowing the relative proportions of these in the gas used nor their relative effectiveness in inducing leaf epinasty. The effectiveness was so low that it was not considered germane to the problem to study the effect of the several isomers.

In the olefine series (ethylene, propylene, and butylene) the effectiveness in inducing epinasty fell rapidly with the increase in number of carbon atoms in the chain. Knight and Crocker (12, p. 362, table) showed three stages of response in the sweet pea seedling which appeared in order with the rise in concentration of the gases: reduced rate of growth, declination of the seedling from the vertical, and horizontal nutation along with swelling. The second response was chosen for column two of Table II, because it required the lowest concentrations of the gases necessary for producing a response that could be determined with certainty without a check plant under exactly the same condition as the gassed plant. An examination of the table shows that the effectiveness of the gases in producing declination of the sweet pea seedling fell in the same order as their effectiveness in producing epinasty of tomato petioles except for propylene which was as effective as acetylene in producing the latter response and only one-fourth as effective as acetylene in producing the former response. Such an exami-

nation will also show that both absolutely and in comparison with ethylene the three gases (acetylene, propylene, and carbon monoxide) were considerably more effective in causing epinasty of the tomato petiole than in producing declination of the sweet pea seedling. Butylene was not tested with the sweet pea seedling.

The petioles of a number of other plants were fully as sensitive as the petioles of tomato in giving the epinastic response to ethylene. The same proved to be the case with the epinastic response to acetylene, propylene, carbon monoxide, and butylene in so far as tested. Fifty parts per million of acetylene or propylene induced evident epinastic response in the petioles of buckwheat, African marigold, golden cosmos, and *Chenopodium album;* 500 parts per million of carbon monoxide acted similarly on the petioles of the sunflower, buckwheat, African marigold, golden cosmos, and *Chenopodium album;* and 50,000 parts per million of butylene induced epinasty in the petioles of the African marigold and *Chenopodium album.*

With the older fractional absorption and combustion methods of gas analyses it was not possible to determine quantitatively the several unsaturated hydrocarbons in illuminating gas. The newer method (9) of fractional distillation of the gas that has been frozen out by liquid air followed by the analysis of the several distillation fractions by the old methods makes the quantitative determination of the several unsaturated hydrocarbons possible. Yant and Frey (22) give such analyses of several sorts of illuminating gas. The percentage by volume of the mixed water and coal gas that they analyzed was as follows for the constituents that produce epinasty in petioles: carbon monoxide 13.25 per cent; ethylene 6.05 per cent; propylene 0.60 per cent; butylene 0.11 per cent. The acetylene was not determined but let us assume that the acetylene percentage was about one-third that of butylene as is the case in coke-oven gases (1, p. 133, table). Now, assuming that one by one each of these constituents was the only one in the gas that would induce petiole epinasty and that in each case the others were displaced by nitrogen, what is the minimum number of parts per million of this gas necessary for each to give epinasty of the tomato petiole? The answer, in round numbers, is as follows: ethylene 1.6 p.p.m.; carbon monoxide 3770 p.p.m.; propylene 8330 p.p.m.; acetylene 136,700 p.p.m.; and butylene 45,400,000 p.p.m. If the gas was used in a concentration just sufficient to make ethylene effective in producing epinasty, carbon monoxide would have about 1/2360 sufficient concentration to be effective; propylene 1/5200; acetylene 1/85,000; and butylene 1/28,400,000. From this it is evident that such a gas would have to be used in very high concentrations to make any constituent except ethylene effective in producing epinasty of tomato petioles and that it would be impossible to have the gas sufficiently concentrated to make butylene effective.

Of the 38 gases and vapors, many of them constituents of illuminating

gas, that were tested as to their power to induce epinasty of tomato petioles, only five were effective: carbon monoxide and four unsaturated hydrocarbons (ethylene, acetylene, propylene, and butylene). Of these five, ethylene was many hundred times more effective than any of the rest, whether figured on the absolute basis or on the basis of their percentage in illuminating gas. This is in agreement with the results of Knight and Crocker (12) in inducing declination of the sweet pea seedling, except for butylene which they did not try.

Flury and Zernik (8, p. 195) state that natural gases contain carbon monoxide and that Carpathian natural gas contains both carbon monoxide and olefines (8, p. 462). Basing their conclusions on analyses of natural gases from many parts of the United States, Burrell, Siebert, and Jones of the United States Bureau of Mines (3, p. 81–82) believe that natural gases do not contain either carbon monoxide or olefines. They suggest that the European workers based their conclusions on erroneous analyses; fuming sulphuric acid and cuprous chloride used respectively to absorb unsaturated hydrocarbons and carbon monoxide will also absorb higher members of the paraffin series, hence the error. The delicate plant test gives a means of answering this disputed question. The Carbide and Carbon Chemical Corporation furnished us with liquefied natural gas from their Charleston, West Virginia wells. This was compressed into a tank that had never been used for other purposes. This gas produced evident epinasty in tomato petioles in concentrations as low as 1 part of the gas to 100 parts of air. The response was probably due to ethylene, acetylene, propylene, or carbon monoxide in the natural gas. In what concentration would each have to exist in the natural gas to give the epinasty with 1 part of the gas to 100 parts of air? Based on the data given in Table II the answer is: ethylene 0.001 per cent or 1 part in 100,000 of air; propylene and acetylene 0.5 per cent; or carbon monoxide 5 per cent. It is quite evident that the last three could not have caused the epinastic response, for if any of them existed in natural gases in such concentrations they would be easily determined by ordinary analytical methods and there would be no dispute about their presence. Ethylene was no doubt the effective constituent. This does not prove that the other three were not in the natural gas in low concentrations. This proves that at least one natural gas contains ethylene and suggests the possibility that others or all may contain olefines and carbon monoxide. Ordinary gas analysis methods are not sufficiently sensitive for determining such low concentrations of ethylene in natural gas.

5. *Use of test plants for detecting ethylene.* Knight and Crocker (12) recommended the sweet pea epicotyl as a test plant for ethylene. The epicotyls were grown in darkness to a height of three cm. and then transferred to the air to be tested and kept in darkness for three days. Since reduction in rate of elongation, which occurred in 0.1 p.p.m. of ethylene, is hard to deter-

mine because of the difficulty of having checks in exactly the same conditions except for presence of ethylene, it is not a convenient response to use. Declination occurred in 0.2 and declination and swelling in 0.4 p.p.m. of ethylene. These are both characteristic responses produced only by unsaturated carbon gases and by these, except ethylene, only in high concentrations. If this is used as a test the concentration has to be at least 0.2 and

FIGURE 4. Bonny Best tomato plants both in Wardian cases 48 hours just previous to photographing: 1. In air; 2. In 0.1 p.p.m. of ethylene.

the exposure three days. Harvey (10), Doubt (7), and Crocker (6) recommended the epinastic response of the leaves of various plants as a good means of detecting ethylene in low concentration. The first two found that castor bean, *Salvia*, *Datura*, and some other plants showed epinastic response in 0.1 p.p.m. of ethylene. Harvey does not state the duration of exposure but Doubt used two or more days. In our work we have found tomato plants especially good test plants for they react quickly and are sufficiently sensitive. In concentrations as high as 0.4 p.p.m. of ethylene the response begins in three or four hours and is complete in 24 hours. Even in 0.1 p.p.m. for 48 hours the epinasty is evident as shown by Figure 4.

Figure 5 shows the epinastic response of six different varieties of tomato to illuminating gas. Two other varieties were tested, Stone and Fruit. All eight varieties gave epinasty in 0.25 p.p.m. of ethylene and all but Fruit in 0.2 p.p.m. of ethylene. All were found sensitive enough to make good test plants, but Bonny Best and Marglobe were found most convenient to use because of their high sensitiveness and the speed and striking nature of

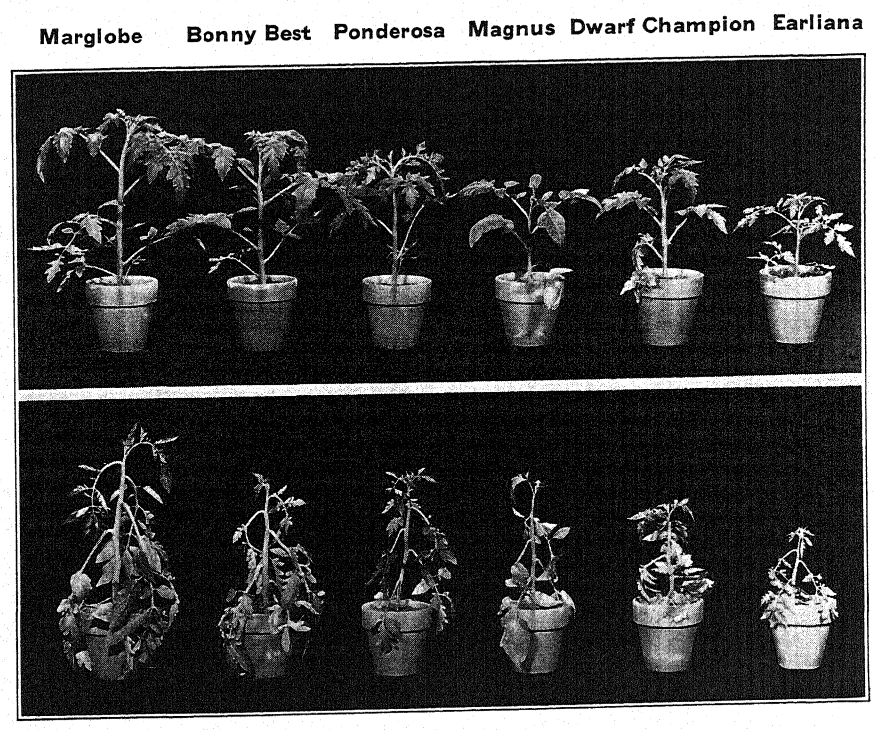

FIGURE 5. Six varieties of tomato. Top row photographed just before exposure to ethylene. Bottom row photographed just after exposure to 1 part of Yonkers gas to 10,000 of air for 40 hours.

their response. All of the plants tested showed increase in sensitiveness and speed of response with increase in vigor of growth.

Certain precautions are necessary in using the epinastic response of leaves of the tomato or other plants in detecting ethylene. Young vigorously growing plants should be used because they are more sensitive and respond more quickly. The upper side of the petioles should all form acute angles with the stem. Whenever possible check plants should be run in air known to be free from ethylene. The temperature should be 15° C. or higher to permit of growth. The test should be terminated after one or at most two days to avoid the long continued effects of other adverse factors.

The declined position of old petioles on mature or nearly mature plants that have been growing for a long time in a greenhouse is no indication

FIGURE 6. Castor bean plant showing the declination of the old petioles without the action of unsaturated carbon gases.

that gas is or has been in the greenhouse. Old petioles of many mature plants have a declined position as is well shown by the castor bean plant in Figure 6. This plant grew in the country where there was no possibility of ethylene influencing it. The same thing has been observed for the old petioles of tomato, papaya, and other plants. Several factors may play a part in the declination of old leaves of maturing plants. Adjustment to light is one. Repeated wilting and revival leads to a declination of older petioles. Age itself is likely a factor. Even previous to blooming the younger petioles of the sunflower, tobacco, *Salvia*, and tomato form a more acute angle with the stem than the old ones. If the young petioles of turgid plants known to be ethylene-sensitive were found in a declined position in a laboratory or greenhouse, one would be justified in suspecting the presence of ethylene. The correctness of the suspicion could be settled by the tests mentioned above.

Wallace (21, p. 537) found that 0.01 p.p.m. of ethylene would produce intumescences on apple twigs if it was allowed to act for sufficient time. This is the most sensitive tissue to ethylene stimulation reported to date. We have found, however, that the leaves of vigorously growing young buckwheat plants show marked epinastic response in 0.05 p.p.m. of ethylene within 12 hours. The cotyledons of this plant are especially sensitive. The third or fourth pair of leaves from the top on vigorously growing young plants of *Chenopodium album* show epinastic growth in 0.05 p.p.m. of ethylene within 12 hours. Figure 9 shows the response of the leaves of the African marigold to higher concentrations of ethylene. All but the top two or three pairs of leaves of the African marigold and the third and fourth pair of leaves from the top of the Golden cosmos respond to 0.05 p.p.m. of ethylene. When considered as partial pressure 1 part of ethylene in 100,000,000 of air is a very high dilution, but when considered as number of molecules per volume the impression is different. In this low concentration of ethylene in air there are still six quadrillion (6×10^{15}) molecules of ethylene in 22.4 liters of space, or about two hundred fifty billion (2.5×10^{11}) per cubic centimeter.

Destructive distillation or incomplete combustion of carbon compounds produces ethylene, carbon monoxide, and other carbon gases. As a result there are several sources of ethylene in the air: illuminating gas; exhaust from automobiles and other gas engines; furnaces of homes and manufacturing plants, when the oxygen supply is inadequate; improperly trimmed oil stoves or torches; a burning brush or rubbish pile; and others. There are two known natural sources of ethylene. It is given off by coal (4) in the mine or in storage and it is present in at least one natural gas. The epinasty of the tomato petiole might, if the occasion demanded, be used to test for ethylene from any of these sources.

In testing for ethylene in a greenhouse or a heated room, the plant is

merely set in the air to be tested and all ventilators and windows closed so that ethylene, if present, will be held about the plant. If ethylene is present the plants will respond in 24 to 48 hours. Ethylene in a sewer or mine can be detected by setting the plant in the sewer or mine if the temperature is high enough to permit response. If not, the plant is sealed in a can provided with an inlet and outlet tube, proper tubing, and a suction pump. The air to be tested is drawn through the can until it displaces much or all of the air sealed in the can. The inlet and outlet are then sealed and the can set in

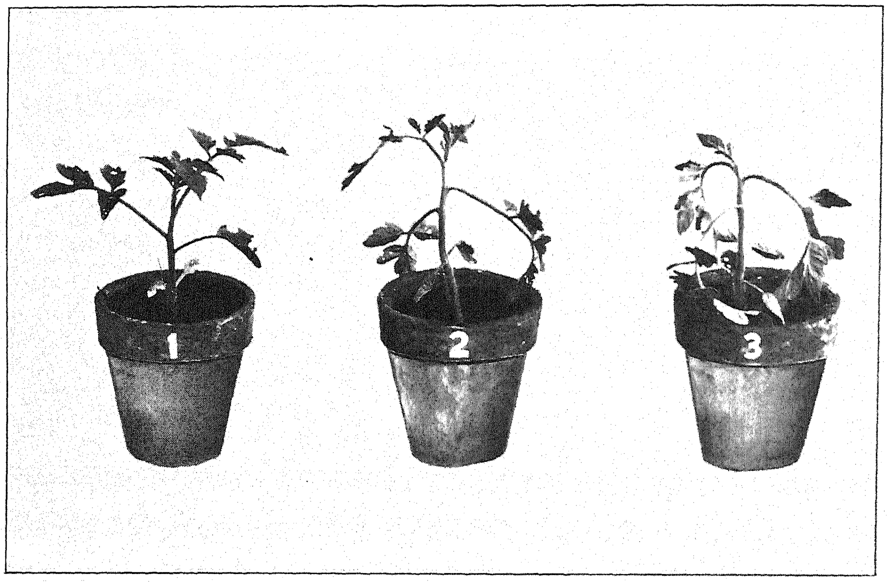

FIGURE 7. Tomato plants used to test for ethylene at a gas line valve pit. Plants exposed 24 hours. 1. Not covered and sitting over a crack of the pit platform; 2. Same as 1 but covered with a bell jar; 3. On the bottom of the valve pit.

a favorable temperature for response until time for examination 24 or 48 hours later. Similarly slight leaks in a gas main can be detected by setting the plant in a post hole near the suspected leak and covering the hole tightly with a board and soil, or if the temperature of the soil is too low, by using the sealed can method.

A coal pile was tested for ethylene production by placing a tarpaulin over the pile and placing a tomato plant under a portion of the tarpaulin that was propped up. Epinastic response of the leaves occurred within 24 hours. The units about a gas and coke plant that give off ethylene were determined by the following method. Vigorously growing potted tomato plants were clamped on the inside of the lids of 50-lb. lard cans; a collar of modelling clay was arranged around the outside of the top of the can; the

can was swung back and forth to fill it with the air to be tested and inverted over the lid bearing the tomato plant and sealed. The cans were set in a room of proper temperature and examined 24 hours later. Charging hot ovens with coal gave off ethylene. Pushing the finished glowing coke from the oven as well as quenching, or heavy spraying, of the red hot coke with water did not produce ethylene. All of the units about the plant were tested in this way and all sources of ethylene located. In the case of units that gave off ethylene, tests were made at various distances in the direction toward which the wind blew to see how far determinable concentrations of ethylene were carried. Ethylene was never detected more than two or three hundred feet from any unit that gave it off. The moving air proved very effective in diluting and dispersing the ethylene.

One test conducted at this plant was of especial interest. A gas line valve pit covered with three loosely fitting planks was tested for ethylene (Fig. 7) by setting one tomato in the pit, one over a crack on the platform with a bell jar covering the plant, and the third plant over a crack on the platform but not covered with a bell jar. The plant on the platform without a cover showed no respone. The other two showed very evident response. Ethylene being only slightly souble in water does not accumulate in the petiole to a great degree with repeated short exposures such as the open air permitted. Ethylene does not injure the plant but induces the reaction through stimulation of growth. Experiments showed that one exposure of two hours even to high concentrations of ethylene did not later produce epinasty. Also the ethylene-induced epinastic growth continued only while the gas was in contact with the petiole for after removal from the gas recovery started as promptly as response began. It is quite different with sulphur dioxide which is very soluble and readily kills plant tissue. Even a few minutes of exposure to this gas will kill intervenous tissue ofthe leaf blade if the concentration is sufficiently high. Also repeated very short exposures will cause killing because the high solubility of the gas leads to its accumulation in the leaf until the lethal dose is reached. Since ethylene has low solubility and the epinastic reaction continues only as long as the gas is in contact with the petiole, tests for ethylene require that the plant be kept continuously in the ethylene for considerable periods. This also accounts for the fact that ethylene effects are seldom, if ever, observed on plants growing in the open.

6. *Ethylene-induced epinasty of leaves as modified by the gravity stimulus.* Since the plagiotropism of various epicotyls growing in air containing ethylene had been shown to be a modified geotropic equilibrium position (12, 16, 17, 19), the question naturally arose whether ethylene-induced epinasty of petioles was a modified response to gravity. Evidence was first sought on this point by exposing plants to ethylene in both the upright and inverted positions. Experiments showed that buckwheat and sunflower plants were

not adapted for such studies because the petioles of these plants when inverted readily righted themselves by torsional growth so that they soon held in part the same position in relation to gravity as the petioles of upright plants. The tomato and African marigold were well adapted to inver-

FIGURE 8. Tomato plants sealed in Wardian cases 24 hours just previous to photographing. A. Intact plants: 1. Upright in air; 2. Upright in 1 part of Yonkers gas to 10,000 of air, 3. Inverted in air; 4. Inverted in 1 part of Yonkers gas to 10,000 of air. B. Decapitated plants: 1. Upright in air; 2. Inverted in air; 3. Upright in 1 part of Yonkers gas to 10,000 of air; 4. Inverted in 1 part of Yonkers gas to 10,000 of air.

sive experiments for the petioles of inverted plants did not right themselves by torsional growth. Figure 8A shows pictures of tomato plants treated in this way. The upright gassed plant showed a marked epinastic response in all the leaves while the leaves of the upright check showed none. The young growing parts of both inverted plants turned upward in response to

gravity. The leaves on the partially reoriented portion of the gassed inverted plant showed marked epinasty, but the petioles on the older part of this plant that remained in the inverted position throughout the experiment showed no epinasty and formed about the same angles with the stem as the leaves on the old part of the check inverted plant. Ethylene-induced epinasty did not occur when the leaves were oriented to gravity in the inverted position.

Attempts were made to avoid the geotropic response of the young portions of inverted plants. In the earlier experiments stakes were set upright in the soil close to the stem and the stem tied to the stakes at short intervals throughout the upper two-thirds of the stem. The stem had to be bound so tightly to the stake in order to keep the plant straight that the binding interfered with the growth of the younger parts of the plant and caused some distortion of both stems and leaves.

Later work showed that cutting off the elongating portion of the plant did not interfere with the response of the older petioles; hence decapitation was used to avoid geotropic response of the tips of inverted plants. Figure 8 B shows decapitated tomato plants exposed to gas in the upright and inverted positions along with checks in air. The petioles of the upright tomato in gas showed decided epinasty, while the petioles of the inverted plant in gas showed no noticeable epinasty and formed about the same angles with the stem as the petioles of the inverted check.

Figure 9 shows the effect of inverting the African marigold upon the ethylene-induced epinasty of the petioles. In the upright plant in air the upper sides of the petioles formed acute angles with the stem. The petioles of the upright plants in 1 p.p.m. and 10 p.p.m. of ethylene showed decided epinasty. The petioles of the inverted plants in both concentrations of ethylene formed about the same angles with the stems as the petioles of the inverted check plants. Inverting the plants caused epinastic response or a more rapid growth on the morphologically upper side of the petiole and this regardless of whether ethylene was or was not present. Two things in connection with the experiment on African marigold need special consideration. In the first place inverted plants were decapitated in order to avoid the confusion that arises from the tops of the plants reorienting themselves to gravity, while the upright plants were intact. Other experiments showed that the leaves on the non-elongating parts of the stems responded to ethylene with the same speed and in the same manner whether the plants were or were not decapitated; hence the decapitation did not modify the results of the experiment. In the second place it will be seen that the petioles of both the check and treated inverted plants showed epinastic response, or grew more rapidly on the morphologically upper side, after inversion. Other experiments showed that the same thing happened to the older petioles of intact inverted plants, hence decapitating

the inverted plants had no effect on the angle of petioles formed with the stems whether the plants were upright or inverted.

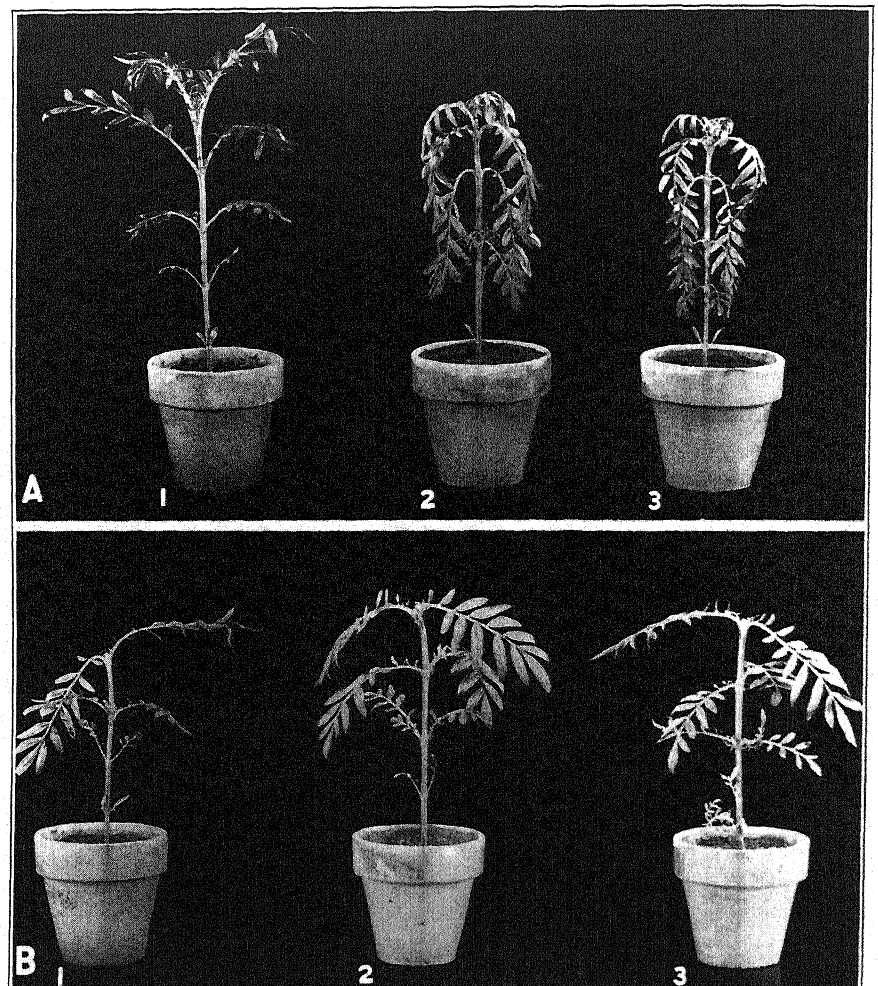

FIGURE 9. African marigold plants sealed in Wardian case 24 hours just previous to photographing. A. intact plants upright: 1. In air; 2. In 1 p.p.m. of ethylene; 3. In 10 p.p.m. of ethylene. B. Decapitated inverted: 1. In air; 2. In 1 p.p.m. of ethylene; 3. In 10 p.p.m. of ethylene.

It has been known for a long time that the inversion of some sorts of plants caused the leaves to grow more rapidly on the morphologically upper side. This was earlier explained by the assumption that inverting the plant removed the restraining action of geotropism and autonomic epinasty was then able to manifest itself.

In discussing the epinasty of petioles of inverted plants, Jost (2) holds that the older interpretation may be wrong and that the response may be merely a geotropic response, resulting from the inverted orientation of the petiole to gravity rather than autonomic epinasty. He also states that for any particular sort of plant the only way to decide the point is to use an intermittent clinostat as Kniep (2, p. 284–286) did for *Lophospermum*.

The conclusion mentioned above that ethylene did not induce epinasty in petioles of the tomato or African marigold when the plants were inverted, was arrived at by observation. It was thought well to test this conclusion by accurate measurements of angle changes of upright checks

TABLE III

CHANGE OF ANGLE IN EPINASTIC RESPONSE OF TOMATO PETIOLES AS SHOWN BY THE ANGLES FORMED BY THE UPPER SIDE OF THE PETIOLE WITH THE STEM

Orientation of plant	No. of leaf*	Angle changed during 22 hrs. in 1 part of Yonkers gas to 10,000 of air			Angle change during 22 hrs. in Wardian case in air		
		Before gassing	After gassing	Difference	Before enclosing	After removing	Difference
No. 1 Upright	1	45°	105°	60°	80°	80°	0°
	2	60°	130°	70°	55°	70°	15°
	3	50°	120°	70°	70°	75°	5°
	4	45°	130°	85°	45°	60°	15°
	5	25°	125°	100°	55°	60°	5°
				Total 385°			Total 40°
				Av. 77°			Av. 8°
No. 2 Upright	1	50°	120°	70°	60°	70°	10°
	2	50°	125°	75°	65°	80°	15°
	3	40°	130°	90°	60°	75°	15°
	4	50°	130°	80°	60°	65°	5°
	5	55°	130°	75°	50°	60°	10°
				Total 390°			Total 55°
				Av. 78°			Av. 11°
No. 1 Inverted	1	60°	60°	0°	60°	70°	10°
	2	60°	75°	15°	60°	70°	10°
	3	55°	65°	10°	60°	60°	0°
	4	60°	85°	25°	60°	65°	5°
	5	45°	70°	25°	45°	65°	20°
				Total 75°			Total 45°
				Av. 15°			Av. 9°
No. 2 Inverted	1	65°	50°	−15°	60°	60°	0°
	2	55°	50°	− 5°	50°	55°	5°
	3	45°	60°	15°	40°	45°	5°
	4	50°	45°	10°	50°	65°	15°
	5	40°	45°	5°	40°	50°	5°
				Total 10°			Total 30°
				Av. 2°			Av. 6°

* All the old imperfect lower leaves were removed. Numbering began with lowermost perfect leaf and ran consecutively upward. The angles of three or four of the younger petioles were not measured because the curvatures were throughout the length of the petioles making accurate measurements impossible.

and treated plants and inverted checks and treated plants. Since tomato petioles show little epinastic response and no torsion when the plants are inverted, tomato plants were used for this study. The angles that the upper sides of the petioles formed with the stems were measured in each case just previously to sealing them in Wardian cases for 22 to 24 hours in the upright and inverted positions both in air and in a gas-air mixture. Table III shows the result of such treatment of eight different plants; two each upright in gas and in air and two each inverted in gas and in air. This table will serve merely to show the method of experimentation and the manner of collecting and recording data. Considerable variation required the study of a much greater number of plants. The work included the study of 20 plants and the determination of change in angle of more than 100 petioles for each condition. The average increase in angle for the gassed upright plants was 56°, for the upright checks 5.5°, for the inverted checks 11°, and for the inverted gassed plants 14°.

The data above show that merely enclosing the plants in a Wardian case for a day produced an average of 5.5° increase in the angle of the upper faces of the petioles formed with the stems. This may or may not have been a significant increase. Either or both of two factors ought to be considered as possible causes of this increase: downward bending of petioles with aging and increased humidity of the Wardian case. Many measurements showed that the upper faces of tomato petioles form less acute angles with the stems as they age but it required many days of aging to give a constantly measurable increase in the angle. Other measurements showed that increasing the humidity did not increase the angle or cause the lowering of petioles. It is very probable that this increase in angle in this experiment was a chance increase growing out of errors in measurements or uncontrolled factors. The 11° increase in the petioles of inverted plants in air was no doubt significant. This is indicated not only by the magnitude of the change but also by the fact that the increase had to occur against the weight of the leaf while in the upright plant it was with the weight of the leaf.

This must be interpreted as either an autonomic epinastic response or a response to the changed orientation to gravity. The petioles of the gassed inverted plants showed an increase in the angle of 14°, or 3° more than the inverted plants in air. This difference was probably not significant and confirmed the conclusion based on observation that gas did not induce epinasty in inverted petioles. The upright gassed plant with an average change of 56° showed decided ethylene-induced epinasty.

Ethylene induced very marked epinasty in the petioles of tomato and African marigold plants when the plants were upright and the morphologically lower sides of the petioles faced the earth. It induced little, if any, epinasty in the petioles when the plants were inverted and the morphologically upper sides of the petioles faced the earth.

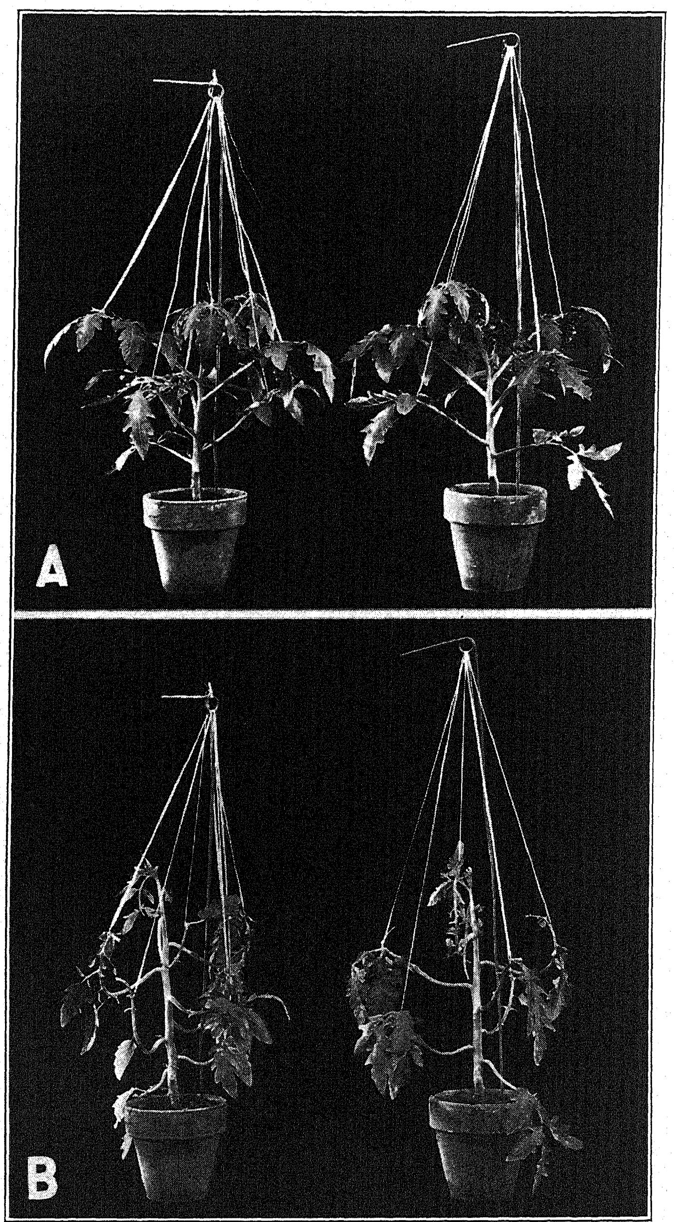

FIGURE 10. Tomato plants with the outer ends of the leaves suspended by means of raffia threads to prevent epinastic response to ethylene: A. Photograph taken just before exposure to gas. B. Same plants photographed after being treated for 48 hours with 1 part of Yonkers gas to 10,000 of air.

Since ethylene caused the epinastic response of tomato petioles when the plants were upright and did not cause it when the plants were inverted, the question naturally arose, was the weight of the leaf a factor in determining the response? Two sets of experiments each repeated several times have shown that the weight of the leaf is not a significant factor in the ethylene-induced epinasty of tomato petioles. The first experiment consisted of holding the outer ends of tomato leaves in their original position while the upright plants were exposed to a gassed atmosphere. The pictures of the two tomato plants at the top of Figure 10 show how the outer ends of the leaves were suspended in position from a wire stake considerably higher than the plants by means of raffia strands just before sealing the plants in Wardian cases with 1 part of Yonkers gas to 10,000 of air for 48 hours. The two pictures at the bottom show the same plants in like orientation to the camera after the exposure to the gassed atmosphere. It is evident that the epinastic response has occurred and because of the inability of the outer ends of the leaves to move downward has caused the petioles to become concave on the upper side. The petioles of the gassed plant are much stiffer than petioles on plants growing in air.

In the second set of experiments the force with which the petioles bend downward was determined. This was done by substituting for the raffia strands, used in the last experiment, delicate wire springs of the type used in the Jolly balance. To avoid the more flexible portions of the leaf the wires were attached to the petiole at a point about two inches from the stem.

The force with which the petioles bent downward was 15 to 30 grams per petiole. The total weight of each leaf was determined later by removal and weighing. The heaviest leaf weighed 3.7 grams; so the epinastic response exerted a force equal to 4 to 8 times the total weight of the leaf.

From the experiment just described it is evident that gravity does not exert its influence on ethylene-induced epinasty through the mere weight of the leaf, but it acts as a stimulus to the complex response mechanism of the petiole. This is proved by the fact that the force of the response is several times the force of the stimulus applied.

A study was made of the effect of rotating tomato plants on horizontal clinostats upon the ethylene-induced epinasty of the petioles. In the first set of experiments the plants were placed with their main axes parallel to the axis of the clinostats as is shown in Figure 11 A, and each clinostat carried eight plants. In the second set of experiments the plants were placed so their main axes were at right angles to the axis of the clinostat as is shown in Figure 11 B, and each clinostat bore four plants. The clinostats made a complete rotation every five minutes and the duration of each experiment was 24 hours. Two clinostats were used in each experiment; one sealed in a Wardian case in air and one sealed in a Wardian case with 10

p.p.m. of ethylene. Plants were also set in the Wardian cases so as to compare the amount of epinastic movements of petioles of upright plants with

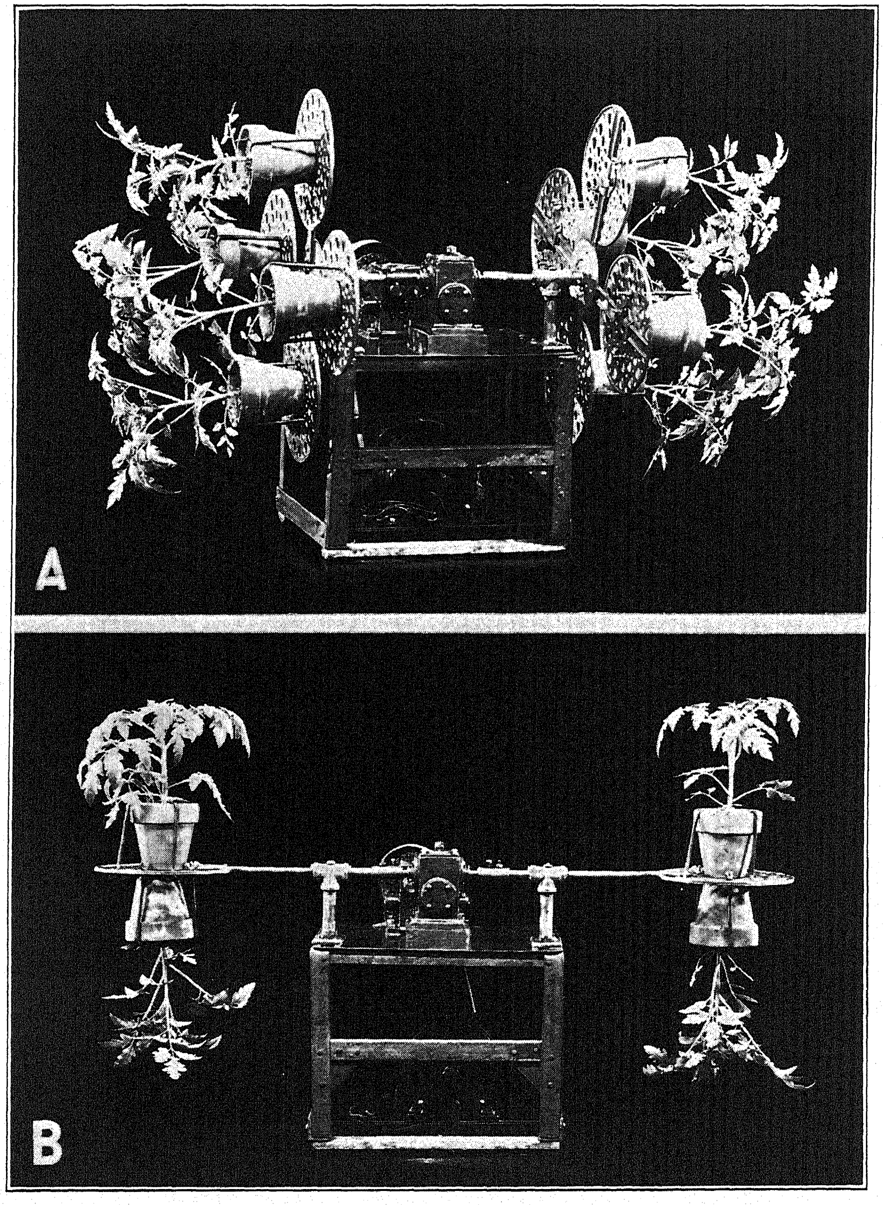

FIGURE 11. Clinostats with horizontal axes used for rotating tomato plants: A. Bearing plants with the axes of the plants parallel to the axis of the clinostat. B. Bearing plants with the axes of the plants at right angles to the axis of the clinostat.

the epinastic movements of rotating plants. The angle that the upper side of each petiole formed with the stem was measured at the beginning and at the end of each experiment and the former subtracted from the latter to get the number of degrees of epinasty shown by the petiole during the treatment.

Table IV shows the epinasty resulting when the axes of the plants were

TABLE IV

EPINASTY OF TOMATO PETIOLES ON A HORIZONTAL CLINOSTAT AS SHOWN BY ANGLES WHICH THE UPPER FACES OF PETIOLES FORMED WITH THE STEM. AXES OF PLANTS PARALLEL WITH CLINOSTAT AXIS. RATE OF ROTATION ONCE IN 5 MIN.

Plant No.	Petiole No. from base upward	Angles in 10 p.p.m. ethylene			Angles in air		
		Before rotating	After rotating 24 hrs.	Difference	Before rotating	After rotating 24 hrs.	Difference
	1	55°	120°	65°	50°	110°	60°
	2	60	120	60	55	90	35
1	3	45	115	70	50	90	40
	4	—	—	—	50	75	25
	1	55	120	65	50	90	40
2	2	60	120	60	50	90	40
	3	50	115	65	50	50	00
	4	40	90	50	45	35	−10
	1	60	125	65	70	110	40
3	2	45	110	65	60	105	45
	3	60	110	50	55	80	25
	4	45	95	50	50	70	20
	1	50	110	60	55	110	55
4	2	45	105	60	45	105	55
	3	60	115	55	50	70	20
	4	40	90	50	55	70	15
	1	55	130	75	60	105	45
5	2	45	100	55	50	110	60
	3	60	115	55	60	115	55
	4	50	100	55	55	55	00
	1	50	120	70	50	80	30
6	2	50	115	65	45	80	35
	3	60	115	55	55	100	45
	4	45	115	70	40	60	20
	1	55	115	60	45	80	35
7	2	65	110	45	50	85	35
	3	45	95	50	45	65	20
	4	50	115	65			
	1	50	115	65	50	85	35
8	2	50	115	65	50	70	20
	3	60	125	65	50	60	10
	4	50	105	55			
Av. epinastic movement of petioles				60°			30°

placed parallel to the axes of the clinostats. The 30 measured petioles on the eight plants rotated in air showed an average epinastic response of 30°. These petioles showed a great variation in the amount of epinastic response varying from −10° to +60°. Judging from this great variation the petioles were in a very unstable position. The 31 measured petioles on the eight plants in the ethylene-air mixture showed an average epinasty of 60°. These petioles showed relatively slight variation in amount of epinasty. The least shown by any petiole was 45° and the greatest 75°. Judging from this, the position finally assumed by the petioles was a rather stable position. Apparently 30° of the epinasty shown by the petioles of gassed plants was attributable to gas itself and the other 30° to rotation on the clinostat. The petioles of the plants sitting upright in the gassed case showed an average of 74° of epinasty and the petioles of the upright plants in air showed an average of 0° epinasty. In the previous portion of this section dealing with the effect of inverting the plants, the average epinasty of the upright plants in gas was 56°. In that case, however, the concentration of ethylene was 3 p.p.m. while in the clinostat experiments the concentration was 10 p.p.m. As would be expected the higher concentration gave the greater response in the 24-hour period.

In the tests with the axes of the plants at right angles to the axes of the clinostats the experiments were run twice in order to give eight plants in gas and eight in air. Otherwise the conditions were the same as described in the previous experiments. Forty-two petioles were measured on the plants rotated in the ethylene-air mixture and they showed an average epinasty of 59° with a variation of individual petioles from 30° to 90°. The same number of petioles was measured on the plants rotated in air and showed an average epinasty of 32° with a variation in individual petioles from −5° to 60°. The petioles of upright plants in gas showed an average epinasty of 77° while the upright checks in air showed an average epinasty of −1°.

It made no difference in the amount of epinasty either in gas or air whether the axes of the plants were parallel with the horizontal axes of the clinostats or perpendicular to the axes. The petioles of the plants rotated in air gave an average of about 30° epinasty while those rotated in 10 p.p.m. of ethylene gave about 60° epinasty. The presence of the ethylene increased the epinasty of the petioles of the rotated plants about 30°. The petioles of upright plants in gas showed epinasty amounting to about 75°. Ethylene induced about two and one-half times as much epinastic movement of petioles of upright plants as of plants rotating on a horizontal clinostat.

When the leaves were detached by cutting the petioles adjacent to the stem of the plants and the leaves were placed in the upright and invert positions in air and in ethylene-air mixtures, the petioles behaved as they

did under similar conditions but attached to the plant. The response of each petiole is independent of the rest of the plant.

All the experiments described in this section of the paper indicate that ethylene-induced epinasty of petioles is intimately tied up with the orientation of the petiole to the direction of the pull of gravity. Ethylene is most effective in inducing epinasty when the plants are upright and the lower sides of the petioles face the earth; it is only 0.4 as effective when the plants are rotated on a horizontal clinostat and only slightly or not at all effective when the plants are inverted and the upper sides of the petioles face the earth. The experiments also show that ethylene in inducing epinasty acts with gravity as a stimulus setting into action the complex response mechanism of the petiole and that the force of the response in upright plants is 4 to 8 times the force of the stimulus.

It appears that ethylene does not induce epinasty directly but that it acts indirectly by modifying the equilibrium position of the petiole with gravity just as it does with pea and other legume epicotyls as shown by Neljubow (16, 17). The situation is more complex with petioles than with orthotropic epicotyls because the petioles are dorsiventral organs and their equilibrium positions vary with the side of the petiole that is downward; the petioles of many leaves when the leaves are displaced from their normal position react with torsive growth which tends to throw the blade of the leaf into the normal position with reference to light or gravity; and finally, the petioles especially in compound leaves like the tomato have a curve in the outer portion of the petiole. The first difficulty can not be avoided but taking the case where the last two difficulties enter in to the least degree, namely the third pair of leaves from the top of the African marigold plant, it will be noted by examination of Figure 9 A-2 and A-3 that as the concentration of gas increased the leaves assumed more nearly the vertical position. In the same way the sweet pea seedling, according to Knight and Crocker (12), declined more and more as the concentration of the gas rose until it approached the horizontal position. Even in the tomato where the outer portion of the leaf forms a considerable curve the main part of the petiole declines more and more as the concentration of the gas increases up to a certain maximum.

DISCUSSION AND SUMMARY

1. Out of the 202 species and varieties of plants tested and reported in this paper, 89 showed ethylene-induced epinasty and 113 did not.

2. Buckwheat, African marigold, sunflower, and *Chenopodium album* showed leaf epinasty in 0.05 p.p.m. of ethylene in the air. Tomato petioles showed a decided epinasty in 0.1 p.p.m. of ethylene in air with 48 hours' exposure. Some plants required much higher concentrations of ethylene. Paper White narcissus, for example, required 3.4 p.p.m. of ethylene. There

was also considerable variation in the type of response shown by the leaves of various plants as Figure 1 shows.

3. In tomato, sunflower, buckwheat, African marigold, *Chenopodium album*, and others, all petioles on the plants, regardless of maturity, showed the epinastic response in low concentrations of ethylene. On some of the plants certain leaves were capable of quicker reaction and response to lower concentrations than others. In Paper White narcissus only the younger leaves showed marked response.

4. In the tomato the upper three or four leaves showed epinastic bending throughout the length or a considerable portion of the length of the petioles. The older leaves bent only near the base of the petioles. When removed from the gas, the young leaves recovered their original position within a few hours, the middle-aged leaves recovered only in part and more slowly, while the oldest leaves showed little recovery. The medium-aged petioles of tomatoes proved especially well adapted to a study of the growth taking place in the response to the gas and recovery from it. As is shown in Figure 3, the response and recovery growth of the medium-aged petioles was limited almost entirely to the basal 6 mm. of the petioles and mostly to the basal 3 mm. of the petioles. Prior to exposure to ethylene these petioles had ceased to grow; so both the response and recovery were due to newly induced growth.

5. Motion pictures of tomato and sunflower plants during response to ethylene and recovery from it showed that normal nutations of the plants were largely stopped by 2 p.p.m. of ethylene in the air but that soon after removal to air the plants resumed these nutations. While ethylene produced growth rigor in the normally growing portions of the plants, it initiated growth on the upper faces of the petioles which gave the epinastic response. The first leaf to show epinastic response in ethylene was the third leaf from the top of the plant, followed by younger leaves and then by successively older leaves. The oldest leaf began its response several hours after the most rapidly responding leaf. The order in which the leaves began their hyponastic recovery after removal to air was the same as the order in which the response took place. The younger leaves recovered completely within 12 hours, the middle-aged leaves less completely after 24 hours, and the oldest leaves showed little recovery. The motion pictures showed that the same concentration of ethylene may act as an anaesthetic on one portion of the plant and as a growth stimulus on another part.

6. Thirty-eight gases were tested as to their ability to induce leaf epinasty in the tomato. Only the following were effective: ethylene, acetylene, propylene, carbon monoxide, and butylene. If the minimum concentration of ethylene required to induce the response was considered as 1, the required concentrations of the others were: acetylene and propylene, 500; carbon monoxide, 5000; and butylene, 500,000. All the effective gases

were carbon gases with unsaturated bonds. In the olefines (ethylene, propylene, and butylene) the effectiveness fell off rapidly with an increase in the number of carbon atoms in the chain.

7. The significance of the unsaturated bond was made evident by a number of facts. Carbon monoxide, an unsaturated gas, was effective while carbon dioxide, a corresponding saturated gas, was not. Ethylene, propylene, and butylene were effective while the corresponding saturated gases (ethane, propane, and butane) were not. Ethylene was effective while ethylene chlorhydrin, a saturated derivative of ethylene, was not. As further test of the significance of the double bond it will be interesting to compare cyclopropane with propylene for both have the same empirical formula, but the first is saturated and the latter has a double bond in the chain. Vinyl bromide, a two carbon atom double bond compound, should be compared with ethylene. Double bond compounds not containing carbon will also be tested. These and other similar tests are to be made as soon as the compounds can be obtained or synthesized.

8. The relative effectiveness of the five gases inducing epinasty is not correlated with their solubilities in water for ethylene is much less soluble than acetylene, considerably less soluble than propylene, and much more soluble than carbon monoxide. Their relative effectiveness may be related to their ability to combine with some constitutent or constituents of the protoplasm.

9. Three unsaturated compounds tested were not effective: acrolein, allyl alcohol, and isoprene. Acrolein and allyl alcohol are respectively the aldehyde and alcohol corresponding to propylene. If the aldehyde and alcohol groups did not modify the effectiveness, they would be expected to cause the response when they constituted 50 p.p.m. of the air. Acrolein killed the plant within 24 hours with 30 p.p.m. in air and did not cause epinasty in lower concentrations. Allyl alcohol killed the plant within 24 hours in 125 p.p.m. of the air, was not toxic in 50 p.p.m. of air, and did not induce epinasty in any concentration. Isoprene was not toxic to the tomato plant when it constituted 3 per cent or less of the air. It did kill the tomato plant in three days when it constituted 10 per cent of the air. It did not induce leaf epinasty in any concentration. The isoprene molecule has five carbon atoms in the molecule with two double bonds in the chain. Judging from the three members of the olefine series, lengthening the carbon chain reduces the effectiveness in inducing epinasty. This may account for the ineffectiveness of isoprene in spite of the two double bonds.

10. The list of the 30 other gases and vapors that were tested and did not induce epinasty can be seen by referring to Section 4 of the Results.

11. The epinastic response of petioles can be used as an extremely delicate test for the presence of ethylene in the atmosphere. While there are a number of plants that give this response to 0.05 p.p.m. of ethylene in the

air, tomato plants of the varieties Bonny Best and Marglobe which respond to 0.1 p.p.m. of ethylene in the air were found best adapted to use as test plants because of the ease of growing and speed of response combined with sufficient sensitiveness for practical purposes. The methods of using tomato plants for detecting ethylene in various places are described in Section 5 of the Results.

12. Ethylene-induced epinasty of petioles was shown to be intimately tied up with the orientation of the petioles to the direction of the pull of gravity. Ethylene was most effective in inducing epinasty when the plants were upright or the lower sides of the petioles faced the earth; it was only about 0.4 as effective when the plants were rotated on a horizontal clinostat; and only slightly, or not at all, effective when the plants were inverted or the upper sides of the petioles faced the earth. Excised leaves behave in the same manner as leaves attached to the plant. The response of each petiole is independent of the rest of the plant. It is certain that ethylene does not act directly in inducing epinasty of petioles but that it acts indirectly, probably by modifying the equilibrium position of the petiole with gravity, as shown by Neljubow for the pea and other legume petioles.

13. In upright plants exposed to ethylene the petioles bent downward with a force equal to 4 to 8 times the weight of the leaf.

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STATISTICAL ANALYSIS OF SEED GERMINATION DATA THROUGH THE USE OF THE CHI-SQUARE TEST

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INTRODUCTION

In an earlier article (12) attention was directed to the fact that the error of an observation may be divided between the error of sampling and the error of estimation. Either of these errors may be negligible in contributing to the errors of the observations. This is true if the error from one source is three times as large as the other. In general a study of the data obtained in an investigation will repay the worker by indicating whether more care should be exercised in sampling or more consideration given to the technique of examining the samples. In many cases the variability of the material is beyond the control of the observer. For example, the varying quantities of arsenic that may be present in a series of samples of six apples each picked from an orchard have to be accepted as facts, regardless of whether the samples happen to be very variable or quite uniform. Nevertheless, a knowledge of the variability of the specimens, whatever it may be, is necessary as a guide for the analyst in the choice of sample size and analytical method.

As an illustration of a case where the only error is the sampling error, we may consider a large reservoir of thoroughly mixed black balls and white balls. If many replicate samples of the same size are drawn and due care observed in counting and recording the observations the variation of the different estimates is solely that of the samples. As is well known, the distribution of the samples may be predicted for any given sample size and percentage of black by means of the binomial expansion. It is possible, therefore, to compute in advance for different percentages an estimate of the precision of a drawing of given size. This device has frequently been used (2, 3, 4, 6, 10) in evaluating data of the general class in which the object is to determine the presence or absence of some particular attribute for every individual comprising the sample. Instances are not lacking, however, where the assumption has not been warranted by the conditions of the experiment. Indeed, it is not safe to conclude that the observational errors are identical with these minimum errors of sampling unless some pains have been taken to establish this fact. If this identity should be established, the laboratory is to be congratulated. Its technique is virtually beyond improvement—only the unavoidable errors of sampling remain and these are simply related to the sample size and percentage so that it is an easy matter to compute them. If, as is more likely to be the case, additional sources of variation are introduced in the course of examining the samples, it is possible to estimate the magnitude of these contributions.

With this information it will be possible to compare the precision of different phases of the work or establish a gradual improvement in precision over a period of time as further conditions are brought under the control of the experimenter. In general, the more involved the procedure required to establish the presence or absence of the attribute in the individuals comprising the sample, the greater will be the difficulty in maintaining an exact set of conditions throughout a series of samples. Since the presence of the attribute in question frequently depends in large part on the very conditions established in the process of examining the sample, strict control of these conditions is necessary. As the number and size of the samples increase, additional obstacles are encountered in providing identical treatment for every individual. In seed germination tests it may be necessary in the course of work with various species to consider many environmental factors. For example, temperature, moisture, light, and fungi, may all have some bearing on the response of the seed. Variation among replicate samples in excess of that indicated by sampling theory is definite evidence of uncontrolled factors or, what is less likely, errors of counting and recording or both. In this paper data on the germination of two kinds of seeds are subjected to analysis with especial reference to the use of the χ^2 test in establishing the uniformity or non-uniformity of large amounts of data. An alinement chart will be described for use in the special case of data which are subject to no other errors than those of random sampling. The paper will also show the necessary modifications in the use of the chart in the case of data uniform in character but containing errors other than sampling errors.

THE BINOMIAL DISTRIBUTION

If p is the probability that a single seed selected at random from a stock is viable, then the terms obtained by expanding the expression $(p+q)^N$ give the various probabilities of obtaining in a sample of N seeds, N viable seeds, $(N-1)$ viable, and so on down to zero viable. In the expression, q equals $(1-p)$ and is the probability that a single seed is dead. Thus if p should be 0.60 the stock contains 60 per cent live seeds. A sample of five seeds may show either 5, 4, 3, 2, 1, or 0 live seeds and the six terms 0.07776, 0.2592, 0.3456, 0.2304, 0.0768, 0.01024 obtained on expanding the expression $(0.6+0.4)^5$ give the probabilities of the six possible events. Now if 1000 samples of five seeds each are selected, the distribution of the samples would be somewhat as follows:

Number viable out of 5 seeds	5	4	3	2	1	0
Number of samples	78	259	346	230	77	10

Although 60 per cent of the seeds are viable, about one sample in 13 will have five viable seeds and about one in a hundred no viable seeds. It should be understood that there is an exceedingly remote chance that the exact theoretical distribution given above would ever be obtained even with a

perfect technique for examining the samples as would be the case if the viability could be determined with certainty on mere inspection. Some degree of divergence in the actual distribution is expected. One of the most valuable contributions to statistical theory has provided the biometrician with a measure of the latitude that may reasonably be allowed for the observed distribution. Naturally enough, if an observed distribution departs markedly from the theoretical the likelihood of its arising through the mere process of sampling becomes very small, and the conclusion follows that the discrepancies are due to extraneous errors which have their origin in the procedure of evaluating the sample. These are the errors which are of particular interest to the technician. It is his task to eliminate them or at least reduce their magnitude. It is not inconsistent to use the word "error" to designate those deviations which may arise through inequalities in the environment of the replicates. The effect of these inequalities is to spread the results over a wider range than that predicted by the theory of sampling. That is, sampling error accounts for part of the spread; errors of estimation, whether these arise from the environment or mistakes in counting, must account for the additional dispersion. Consequently the technician will appreciate the helpfulness of a procedure which can be employed to demonstrate the presence or absence of these additional errors, and furnish an estimate of their size.

Only in an experiment especially designed for examining the agreement of an observed distribution of seed samples with the binomial distribution would one be likely to find an adequate number of replicates under identical conditions. In practice it is more common to find associated with a large number of samples an equally large number of conditions so that no replicates are available. In this case no analysis of the data is possible. If the seeds under each set of conditions have been divided into two portions (preferably but not necessarily equal) and the results recorded for each half, the situation is entirely changed. This would ordinarily not greatly increase the labor of the experiment, but it makes a great deal of difference in the matter of investigating the reliability of the data.

THE CHI-SQUARE TEST

The χ^2 test is used to compare the distribution of frequencies realized in an experiment with the distribution predicted from some hypothesis. The difference between the number found in any class and the number predicted is squared and divided by the number predicted. This process is extended over all the classes, and the results summed. The total is the value of χ^2. The smaller the value of χ^2 the better the agreement of the expected and actual frequencies. Pearson (8) in 1900 established the nature of the χ^2 function and made it possible to calculate the probability that any value of χ^2 will be exceeded. Therefore, from tabulated values of the χ^2 function

the odds may be determined that a particular value of χ^2 derived from a set of data could arise in the process of sampling. Values of χ^2 are given for different values of n where n is usually one less than the number of frequency classes involved in the comparison. Thus in the case of the distribution of the 1000 samples mentioned earlier, if an actual experiment were available, χ^2 could easily be computed for the six frequency groups. The table of χ^2 given by Fisher (5, p. 96) entered with n equal to 5 shows that χ^2 is greater than 11.07 in only 5 per cent of the cases and less than 1.145 in 5 per cent of the cases. Hence if the computed value is not larger than 11.07 (nor less than 1.145), it is concluded that the observed distribution does not deviate unreasonably from the expected.

As has been pointed out, laboratory practice does not usually afford many replicates under the same conditions. If the number of replicates is too small to construct a frequency distribution, χ^2 may be calculated from the data by the formula, given by Fisher (5, p. 70),

$$\chi^2 = \frac{S(x-\bar{x})^2}{pqN} \qquad \text{(Formula 1)}$$

where N is the number in each replicate, p the fraction viable, q the fraction dead and $S(x-\bar{x})^2$ the sum of the squares of the differences of each sample from the mean. The χ^2 table is entered with one less than the number of replicates.

If a number of series of replicates, even under different conditions, are available the values of χ^2 from each series may be summed and the total value examined. For example, if six series of ten replicates each are totaled n becomes 6×9 or 54. Since tables of values of χ^2 for large values of n are not available the result is interpreted as follows. The value of the expression $\sqrt{2\chi^2}-\sqrt{2n-1}$ is found, and if not numerically larger than 2 the value of χ^2 is not unreasonable and the distribution of the data may be concluded to have arisen through the process of random sampling. (When n is large the expression $\sqrt{2\chi^2}-\sqrt{2n-1}$ equals zero with a standard deviation of 1.) If, as is frequently the case, each series consists only of duplicates, the same process is carried through. The value of χ^2 from a pair of duplicate samples may be found by Formula 1 or by constructing a fourfold table (5, p. 83; 7, p. 317). In the latter case χ^2 may be calculated even though the duplicate samples are not the same size.

In the case of duplicate samples it is likely that a large number of pairs will be available. In that event the various values of χ^2 obtained may be examined to see if they are distributed in accordance with the χ^2 function. Thus, for n equal to one, the χ^2 table shows that 10 per cent of the values of χ^2 will fall between zero and 0.0158, 10 per cent between 0.0158 and 0.0642, 10 per cent between 0.0642 and 0.148, and so on. If a considerable number, as 100 or more, of χ^2 values have been computed they may be so grouped on the basis of their magnitude. The numbers falling in each group

may then be compared with the expected distribution. A measure of the agreement between the two distributions is afforded by applying the χ^2 test in the manner described in the beginning of this section.

DATA AVAILABLE FOR EXAMINATION

The data utilized in this paper are taken from the original laboratory records of an extensive series of tests on the vitality of delphinium seeds when stored under various conditions. The results of this work have been reported by Barton (1) in this Journal and include over 800 tests in duplicate samples of 100 seeds each. Such an array of data, based on more than 160,000 seeds, provides a substantial basis for an investigation to determine whether or not the samples follow the binomial distribution.

The data on the germination of wheat seeds under water have been taken from a recent paper by Tang (11). This paper deals with the response of the seeds at different temperatures and varying degrees of aeration, when submerged beneath a nutrient solution for definite time intervals. In all 168 sets of conditions were arranged. Samples of 100 seeds were selected and the entire work replicated five times. A total of 84,000 seeds were used in these experiments which were especially designed to bring out the influence of important environmental factors on the germination of seeds. No statistical analysis was presented by the author but it may be shown by the methods here employed that the different runs varied greatly in the degree of agreement shown by the replicates.

EXAMINATION OF THE DATA

The number of replicates available under a given set of conditions is two in the case of the delphinium seeds, and five in the case of the wheat seeds. Even five replicates are altogether too few to form a distribution to determine directly if the counts are distributed in a binomial series. It is necessary, therefore, to compute χ^2 by Formula 1 for each set of replicates. These computed values of χ^2 may vary greatly and individually do not afford a very good test. The sum of a series of χ^2 values is much better, and, if a sufficient number are available, the distribution of the computed values of χ^2 provides the best means of ascertaining if the variation among the replicates is simply that of random sampling. The various steps in this procedure will now be given in detail.

If two samples of 100 seeds each show 55 and 63 seeds germinating respectively, the mean is 59 and the difference of each sample from the mean is 4.

$$\text{Then } \chi^2 = \frac{(4)^2+(4)^2}{100(0.59)(0.41)} = 1.322$$

This calculation must be made for every pair of samples, and the values of χ^2 tabulated. The values of χ^2 range from zero (when the samples are the

same) to several units. The distribution of the values of χ^2 which arise from random sampling is known. In column 1 of Table I certain values of χ^2

TABLE I

DISTRIBUTION OF χ^2 VALUES COMPUTED FROM 709 DUPLICATE SAMPLES OF 100 SEEDS EACH

χ^2	Per cent expected	Observed distribution 709 values	Expected distribution 709 values	Distribution adjusted* χ^2 values
0				
	10	43	70.9	74
0.0158				
	10	49	70.9	77
0.0642				
	10	67	70.9	62
0.148				
	20	118	141.8	159
0.455				
	20	138	141.8	145
1.074				
	10	51	70.9	62
1.642				
	10	85	70.9	59
2.706				
	5	51	35.5	34
3.841				
	3	40	21.3	24
5.412				
	1	20	7.1	1
6.635				
	1	47	7.1	12

* The χ^2 values calculated by Formula 1 were divided by the factor 1.9. See p. 229.

(for $n=1$) are listed. In column 2 the first entry gives the percentage of χ^2 values which fall in the range zero to 0.0158, the succeeding entries give the percentages of the χ^2 values which lie within the limits shown in column 1. Column 3 shows the distribution of χ^2 found with 709 pairs of samples, and column 4 the expected distribution of 709 items. The entries in column 5 will be discussed in a later paragraph. The most marked deviations from the expected distribution occur for the large values of χ^2. All the values of χ^2 have now been sorted into 11 classes and the χ^2 test may be utilized in comparing the observed distribution of χ^2 among the classes with the expected. The difference between corresponding entries in columns 3 and 4 is squared and divided by the expected value. (The figures in the last two horizontal rows of the table are combined into one class before taking the difference between the observed and expected frequencies. The use of the χ^2 test in comparing frequency distributions requires in general that no class be smaller than 10.) The total, more than 200, for the ten classes constitutes χ^2 for this comparison. The table of χ^2 shows that, with n equal to 9, only once in 20 times will χ^2 exceed 16.9. Since the value of χ^2 found is

much greater than this the discrepancy between the two distributions is real and not a matter of sampling. The values of χ^2 computed from the duplicate samples are consequently not in accord with those which might arise in the process of sampling but have been influenced through the introduction of other errors. The same conclusion might have been arrived at more quickly by simply summing up the 709 values of χ^2 from the 709 pairs of samples. The total is 1348.3 and n is 709. The total may be tested by evaluating the expression $\sqrt{2\chi^2}-\sqrt{2n-1}$. Substituting the values for χ^2 and n gives $51.9-37.6$ or 14.3. The difference is definitely outside the range -2 to $+2$ between which limits the value of the expression will fall about 95 per cent of the cases if sampling errors alone are present.

The examination of the data so far indicates the presence of errors other than those of sampling in the observations. It will be profitable to continue the examination of the data when divided into sections. The data were accumulated in the course of 20 experiments, several workers assisting in securing the data. A division of the data by experiments is a natural one and might disclose some unevenness between different portions of the work. Another division of the data may be based on the six different seed crops. The data may also be divided into the annuals and the perennials. There is no reason to expect any differences in precision between groups classified on the basis of seed origin, unless some particular seed crop should be more responsive than the others. In the event that germination conditions were not maintained exactly for the duplicates greater differences might show in the more sensitive lot. Four different sets of conditions were used for storage, each crop being stored in an open container and in a sealed container in each environment.

Table II presents a concise summary of the χ^2 tests made on the data classified by experiments, crops, and storage environment. The table lists the number of pairs of samples in each group, and the total χ^2 for the members of the group, and the result of the test to determine if the value of χ^2 falls within reasonable limits. Among the 20 experiments (listed in order performed) 6 are found to have values of χ^2 which might arise through sampling errors alone. This is not necessarily proof that these experiments are definitely of a different order of accuracy from the rest. Actually the precision of the observations, as established by all the data, is close enough to the sampling deviations, so that small groups may not disclose the presence of other errors. About one-third of the groups of 30 odd pairs may be expected to appear satisfactory. In the neighborhood of 60 pairs are necessary to insure that a group, although really including introduced errors of the magnitude found in the data, will not appear more often than once in 20 times to have sampling errors alone. The two experiments giving χ^2 values greater than seven are definitely below the general performance. Where the data are classified into larger divisions, by seed crop or by stor-

TABLE II

χ^2 TESTS ON DIFFERENT PORTIONS OF THE DELPHINIUM DATA WHEN GROUPED BY EXPERIMENTS, CROPS, AND STORAGE CONDITIONS

Data grouped by experiments		
No. in group	Total χ^2	$\sqrt{2\chi^2}-\sqrt{2n-1}$
34	79.4	4.42
40	63.0	2.33
42	52.5	1.15
39	68.3	2.92
41	72.1	3.00
30	123.4	8.02
38	81.5	4.11
37	61.2	2.52
38	81.8	4.13
37	35.9	−0.08
38	99.7	5.45
37	58.5	2.26
36	83.0	4.46
33	47.5	1.69
33	38.6	0.73
33	123.9	7.69
31	59.1	3.06
32	35.1	0.44
31	35.5	0.61
29	48.1	2.26

Data grouped by crops		
No. in group	Total χ^2	$\sqrt{2\chi^2}-\sqrt{2n-1}$
Annuals		
143	320.7	8.4
155	325.2	7.9
152	333.4	8.4
Perennials		
10	11.3	—
112	148.5	2.3
137	209.1	4.0
Data grouped by previous storage		
No. in group	Total χ^2	$\sqrt{2\chi^2}-\sqrt{2n-1}$
Open storage		
58	119.1	4.7
78	206.1	7.8
99	142.1	2.8
98	160.8	3.9
Sealed storage		
83	138.0	3.8
98	210.8	6.5
99	201.4	6.1
96	169.9	4.6

age, none appear to have errors of sampling alone. Since it developed that sealed storage conserved the vitality of the seeds much better than open storage, the data for each crop were divided again to contrast the open and sealed storage. It is through such classifications of the data that lack of homogeneity in the whole work may be disclosed if present.

In Table II the first three groups listed under crops are annuals, the other three groups are perennials. The crops are those of 1924, 1925, and 1926 and are given in that order. Very few seeds germinated from the 1924 perennial crop. In this group, as with the rest, all cases in which both samples of a pair showed zero germination were eliminated. The perennials appear to deviate less from sampling theory than the annuals. To find if this was only an apparent difference, the distribution of the χ^2 values for the perennials was tabulated, just as was done for the entire data in Table I. The distribution of the χ^2 values furnished by the annuals was also listed.

The two distributions were then examined to see if they represent different populations. This test, devised by Pearson (9), will not be given here but may be found in the works of Pearl (7, p. 322) or Fisher (5, p. 85). The conclusion is drawn that the evidence is on the border line of showing a significant difference in accuracy between the data obtained with the perennials and the data pertaining to the annuals. In general the perennials showed lower percentages of germination than the annuals. The further division of the data on the basis of storage shows no contrast between the results with open and sealed seeds.

It is appropriate to consider the interpretation of the data in the light of the information gleaned by the statistical analysis. In this case it appears that the very nature of the study of the vitality of seeds involved about 20 repetitions at intervals of a few months and thus established the trend curves with a high order of certainty. There is no difficulty, therefore, in drawing sound conclusions concerning the vitality under different storage conditions without resort to a detailed statistical analysis. The chief contributions made by such an analysis lie in its use in detecting the sources of error in the experimental work and therefore directing attention to their elimination, and in the comparison of the precision of different procedures, as for example, tests in the greenhouse and in the laboratory. The large volume of data available on these seeds dictated their selection to illustrate the various steps in the statistical analysis, although, for reasons about to be given, the estimate made of the errors does not do justice to the work. The size of the sample selected for this work was 200 seeds and the division of these into two petri dishes each with 100 seeds was a matter of convenience. Inasmuch as the total of the germinated seeds was all that was required, no special efforts were made to insure that the assistants, in recording the data from time to time in each petri dish, kept the two strictly separate in the record. More recent studies with these seeds indicate a much closer agreement with the binomial distribution.

Reference has been made to the fact that the errors other than sampling are small enough so that as many as 60 pairs of duplicates are required to insure that an experiment does not appear to have sampling errors only. If triplicates instead of duplicates were available 30 sets would be sufficient since n for each triplicate set is two, and the total for the 30 becomes 60. Thus, fewer seeds would suffice to disclose the precision of the work.

The questions that arise in practice in seed germination tests most often take the form of queries as to the significance of the difference in percentage germination found under diverse conditions. On the assumption that errors of sampling prevail to the exclusion of others the answers are easily found. The probable error of the number of live seeds found in any test is given by the formula

$$(\text{P. E.}) = 0.6745\sqrt{pqN} \qquad \text{(Formula 2)}$$

where p fraction germinating, $q = 1 - p$, N equals the number of seeds in the sample. For example, if 320 seeds germinate out of a sample of 400 seeds, p is 0.80, q is 0.20, and the value of the expression is 5.40. The result may be written either as 320 ± 5.4 or 80.0 ± 1.35 per cent. If the probable errors for two tests are designated by $(P.\ E.)_1$, and $(P.\ E.)_2$, the probable error $(P.\ E.)_d$ of the difference in percentage between the two values is

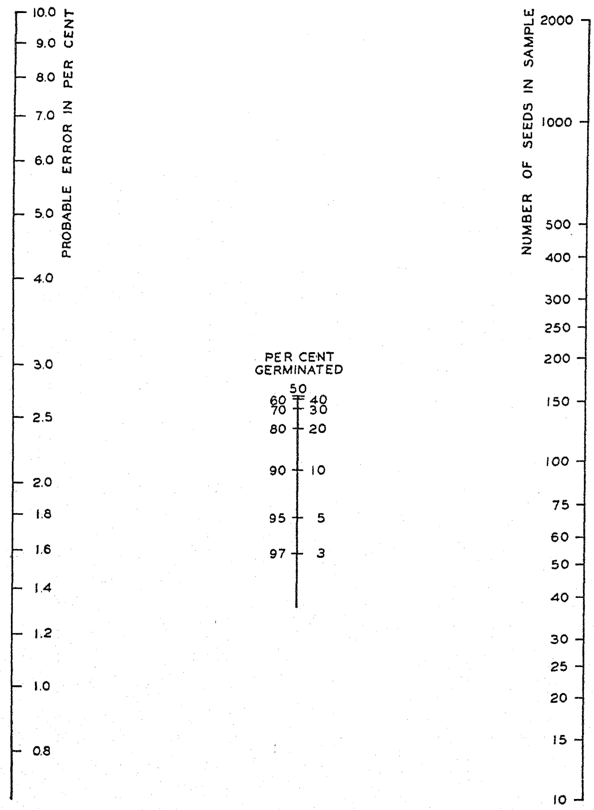

FIGURE 1. An alinement chart for finding in per cent the sampling error associated with any given sample size and percentage germination.

simply $\sqrt{(P.\ E.)_1^2 + (P.\ E.)_2^2}$. The ratio of the difference in percentage to $(P.\ E.)_d$ must equal 3.0 for odds of 22 to 1. If duplicates are available, they are simply combined and treated as one sample. The computation for the probable error of a sample is greatly facilitated by the use of the alinement chart shown in Figure 1. The number of seeds in the sample and the percentage of seeds germinated are located on the appropriate scales and the line joining these points extended until it intersects the probable error scale. The odds are 22 to 1 that the true value of the germination lies

within the limits of plus or minus three times the value for the probable error.

This simple procedure for the interpretation of germination tests is based on the assumption that sampling errors alone are involved. In general this will not be the case. In the illustration discussed above the total of the values of χ^2 was found to be 1348.3 with n equal to 709. It was also found that this did not arise from excessive errors in a localized portion of the work. Since a satisfactory value for χ^2 requires that the value of the expression $\sqrt{2\chi^2}-\sqrt{2n-1}$ fall within the limits of $+2$ and -2, it follows that for large values of n the value of χ^2 is approximately equal to n. In this case the value of χ^2 is about 1.9 times n. Each individual χ^2 was computed from the formula

$$\chi^2 = \frac{2d^2}{100pq}$$

where d is the difference of each sample from the mean of the two samples. Now if the figure of 100 in this formula is replaced by 190 the values of χ^2 have a reasonable total. The distribution of the individual values of χ^2 calculated on this basis is shown in column 5 of Table I and now shows good agreement with the theoretical frequencies listed in column 4. In other words the differences between the duplicates are those which would have been found if samples of 190 seeds had been used in place of 100 seed samples. The probable error of a sample may then be taken as $0.6745\sqrt{190pq}$ instead of $0.6745\sqrt{100pq}$. The ratio of the actual probable error to the probable error of sampling is $\sqrt{190/100}$ or 1.38. The percentage probable errors read from Figure 1 should be multiplied by the factor 1.38 in interpreting the delphinium data. In this case the deviations arising in the course of examining the samples are nearly equal to those due to sampling.

The data on the germination of wheat seeds are given in Table I of Tang's paper (11) and will not be reproduced here. In this table are given the germination counts of five replicate samples of 100 seeds each under 168 different sets of conditions representing every possible combination of seven different temperatures, six different rates of aeration, and four different time intervals. It is a simple matter to calculate (Formula 1) χ^2 for each set of five replicates and tabulate the results. The distribution of the 168 values was examined as in Table I for the delphinium data, the values of χ^2 being taken in this case under $n=4$ from the χ^2 table. The observed distribution was found to deviate significantly from the expected frequencies showing the presence of errors other than those arising in the process of sampling. This is confirmed by the value of 965.8 for χ^2 for all the data where n is equal to 672. An analysis of the data was then made to determine if this excessive value for χ^2 was due to some particular portion of the work, as was done in Table II for the delphinium experiments. Thus, from

the tabulated values of χ^2 the 24 entries corresponding to tests run at 12° C. were summed. The total is the first entry in column 5 of Table III. The

TABLE III

χ^2 TESTS ON WHEAT SEED GERMINATION DATA[1] WHEN GROUPED BY TEMPERATURE, AERATION, AND DURATION

Data grouped on basis of aeration			Data grouped on basis of temperature			Data grouped on basis of duration		
Air flow l./day	χ^2	$\sqrt{2\chi^2}-\sqrt{2n-1}$	Temp. °C.	χ^2	$\sqrt{2\chi^2}-\sqrt{2n-1}$	Hours run	χ^2	$\sqrt{2\chi^2}-\sqrt{2n-1}$
0	82.8	−2.07	12	80.8	−1.11	6	157.7	−0.54
1	116.4	0.32	19	203.2*	6.34	12	184.7	0.92
3	128.7	1.12	24	229.8*	7.61	18	309.1*	6.56
6	128.3	1.09	30	154.1*	3.73	24	314.3*	6.77
15	202.2*	5.17	35	79.3	−1.23			
30	307.4*	9.86	40	102.1	0.47			
			45	116.5	1.45			
n for each group = 112 5% limits for χ^2: upper = 144; lower = 84			n for each group = 96 5% limits for χ^2: upper = 125; lower = 70			n for each group = 168 5% limits for χ^2: upper = 206; lower = 133		

[1] Based on data published by Tang (11).
* Values of χ^2 in excess of those postulated by theory of sampling.

value of n for this total is 24×4 or 96 and the value found for χ^2 for the experiments performed at 12° C. is satisfactory. This process was repeated for all the other temperatures. A comparison may now be made of the precision of the work at the several temperatures using the value of χ^2 as a guide. It is to be noted that the comparison of the results obtained with the different temperatures is not made under just one set of conditions but under 24 sets which enhances the value of the comparison. It is apparent that temperatures of 19°, 24°, and 30° C. show marked discrepancies from sampling theory. In an entirely analogous manner it is disclosed that the runs with different rates of air flow show that the experimental conditions were reproduced less successfully as the rate of aeration was increased. This is not surprising from an experimental point of view. Also the runs of 18 and 24 hours duration show much greater variation among the replicates than the shorter intervals. Presumably if the temperature or other factors were not adequately controlled the longer intervals gave a better opportunity for differential response. It is easy to give an explanation for the higher values of χ^2 obtained at temperatures of 19°, 24°, and 30° C. If the general temperature-germination curve is examined it appears that it is in just this range that the germination is most affected by temperature changes. At the extreme temperatures the curve flattens out on both ends and a change in temperature alters the germination but slightly. It appears

then that in this work the extreme temperatures were controlled or reproduced adequately, but there was room for considerable improvement in the control of temperature and other factors in the temperature range that permitted good germination. The starred values of χ^2 in Table III indicate variation among the replicates concerned, considerably in excess of that postulated by the theory of sampling. It is significant that just those cases are starred where the conditions were favorable for germination and this should focus the attention of the worker on the desirability of expending especial care in those circumstances. No such exacting control of the environment is required in the end conditions which do not favor germination.

DISCUSSION

It should not be concluded that the existence of variation other than that arising from sampling is evidence of careless work. Quite the contrary may be true. The attainment of the ideal, that is, the exact reproduction of a given environment for all of a series of replicates, may involve an amount of labor altogether disproportionate to the gain in accuracy. It is often far easier to use a somewhat larger number of seeds if an increase in precision is desired. The Chi-square test is advanced as an efficient statistical procedure to measure the improvement in accuracy as a result of additional experimental precautions.

The number of seeds in a sample and the number of replicates employed in seed germination tests depend upon the objective in view. More seeds are required to establish a small difference than a large difference in the viability of two lots of seeds. Differences less than some given amount may be unimportant in a particular investigation. Hence there is no advantage in using more seeds than are required to show the existence of this minimum difference. In both sets of data, which have been taken from the papers of Barton (1) and Tang (11), it is clear that the authors used ample numbers of seeds to demonstrate the points brought out in their papers. The present paper shows how additional information regarding their experiments, and perhaps other similar experiments, may be obtained by means of the Chi-square test. And while such an analysis was not required for the establishment of the conclusions which they drew, it is likely that in many experiments such an analysis would be of critical importance for safe interpretations.

SUMMARY

A brief discussion has been presented of the binomial distribution and the use of the χ^2 test to determine if an observed distribution is in accord with the binomial law.

The steps involved in the application of the χ^2 test have been illustrated with data taken from a study of the vitality of delphinium seeds.

Some data on the germination of wheat seed have been taken to show the usefulness of the χ^2 test in disclosing how different portions of an experimental study may vary greatly in accuracy. Although, for example, the temperature may be controlled equally well in a series of runs at various temperatures, the precision of the results may be very different in the several temperatures.

An alinement chart has been arranged for the rapid estimation of errors of random sampling. A procedure has been given for extending the use of the chart to data which have been shown by the χ^2 test to have errors greater than those of random sampling.

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THE PRECISION OF SPORE GERMINATION TESTS[1]

S. E. A. McCallan and Frank Wilcoxon

The germination of fungous spores is an important problem which enters into many phases of plant pathological work. Undoubtedly the greater part of the interest in spore germination comes under either one of the two general fields of life history studies, or of toxicology. In the former the object is primarily to ascertain under what conditions, especially those of temperature, the spores will germinate; while in the latter it is to determine the relative toxicity of two or more compounds. Spore germination might also be employed as a criterion in the identification of species.

In all of these cases the main object is that of a comparison. Is infection more likely to take place at a high temperature or at a low temperature? Is compound A more toxic than compound B? In other words, being interested in comparisons, we are concerned with differences and hence as to whether the differences are significant.

The variations in spore germination are familiar to all who have worked on this problem. Over 30 years ago Duggar wrote, "No studies of importance seem to have been made upon the variation in capacity for germination of individual spores produced under similar conditions. . . . Nevertheless, great individual differences exist, and in any medium which is not a strong stimulus for germination, varying percentages of perfect germination will invariably occur, whatever precautions of method may be observed" (2, p. 64). On the other hand there has been perhaps a feeling that if the technique were truly perfect these variations would disappear.

In the following pages the authors will endeavor to show that the germination of fungous spores is an orderly process with well-defined variations. These variations appear to be of two kinds. One is due to uncontrolled conditions, that is, faulty technique. These may be reduced or eliminated entirely. The other is that of sampling. The sampling variations cannot be eliminated. Thus, it will be shown that when all the conditions are adequately controlled only the sampling variations are of importance. The variations of sampling follow a definite mathematical law; hence they may be anticipated and it becomes a simple procedure to make accurate comparisons to determine if real differences between treatments exist. Likewise the converse is true, that when it has been found that the only variations are those of sampling, the technique of spore germination may be considered to be satisfactory for this purpose.

Some of the studies on spore germination are not as conclusive or convincing as they might be. This is largely because of one or more of three

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common errors: (a) failure to state the number of spores counted; (b) expressing the control germination as 100 per cent and adjusting the treatments accordingly; and (c) giving the results of progressively changing treatments that will not follow a smooth curve. If the number of spores counted is not stated the degree of significance to attach to the result is unknown to the reader. The second error not only hides the real values from other workers, thus preventing adequate comparisons, but is apparently often done from the mistaken idea that by so doing one set of results may be compared directly with another. This is entirely erroneous, for very small differences in control germination will result in large differences in the treated spores (5). The germination of fungous spores is a process which does not vary by jagged steps when plotted against progressively changing treatments. If the temperature or toxicity curve is not reasonably smooth, an insufficient number of spores has been counted or errors have arisen because of uncontrolled conditions.

CONTROLLED CONDITIONS

The first requisite in spore germination studies is that all conditions must be controlled or known as far as possible. The most important factors to be considered here are:—Cleanliness of the glassware, source and age of spores, density of spore suspension, germination medium, concentration of toxic agent, temperature, and time. The experimental procedure adopted should lend itself readily to the germinating and counting of large numbers of spores. The hanging drop method does not meet these requirements. These and other less important factors have been discussed in detail by McCallan in an earlier article (5) and also by Doran (1), Duggar (2), and others and with certain exceptions need not be enlarged upon here. It is sufficient to state that all of these factors are significant and the disregard of any will give unsatisfactory results.

Density of spore suspension. In many cases this factor has not been given the consideration its importance warrants. The concentration of the spores within the drop of water or other germination medium should be determined by actual inspection under the microscope, and in the case of replicate tests should be practically constant and as uniform as possible. In some species the aggregating of spores is difficult to prevent and is a source of error. McCallan (6) has shown that for a given concentration of copper sulphate solution the percentage germination obtained increases with an increase in the density of the spore suspension. A similar effect has been observed with sulphur dust. In the case of toxic agents the greater the number of spores present, the less the amount of toxic agent per spore and hence the higher the resulting percentage of germination.

Concentration of toxic agent. In the case of solutions and suspensions the concentration can be determined with accuracy. The precise determination

of concentration for dusts and gases is more difficult. Toxicity tests are, however, of little value unless the concentration of the toxic agent is known with some degree of accuracy. The authors have shown (12) that it is possible to obtain satisfactory weighings of the sulphur dust present on glass slides as used in ordinary toxicity tests. In some cases, as the authors have further shown (12), the important factor is not the weight of material but the number of particles and in these cases actual counts of the particles may be made with greater accuracy and convenience than the weighings. It is probable that the toxicity of unstable compounds can never be determined precisely because of the uncertainty of the actual concentration during the experiment.

Tests performed at different times. Unfortunately the exact causes for the variations in controls from day to day are yet unknown, and until this problem is solved it is futile to attempt to make direct comparisons of results obtained at one time with those obtained at another. As has been pointed out before, small variation in controls will result in a large variation for any treatment. Thus in replicate experiments performed at different times all that can be expected is that the relative order of toxicity will be the same.

VARIATION OF REPLICATE TESTS

When spore germination tests are performed in drops on glass slides, or in any other manner, it is found that the percentage of spores which germinate varies somewhat in the different drops and it is necessary to consider what degree of confidence may be placed in the final value obtained by combining the results of a number of replicate counts. Even if it were possible to have identical conditions in the different drops, nevertheless some variation would be observed. This residual variation is called the "error of random sampling," and it should be emphasized that no improvement in technique will eliminate it.

The variations or errors of random sampling may be illustrated in this manner. Consider a large bag containing a very large number of seeds of two kinds, black seeds and white seeds. And suppose that 40 per cent of the seeds are black and 60 per cent are white. If now, samples are withdrawn from the bag in lots of 50 seeds, the first sample might contain 22 black seeds and 28 white seeds, the second 19 black and 31 white, and the third 15 black and 35 white. These values are different and none contain exactly 40 per cent black and 60 per cent white, and the true percentage of black seeds and of white seeds in the bag remains unknown. These are variations of random sampling and cannot be eliminated, although they may be proportionately reduced by drawing a large number of seeds. The deviations obtained in experiments of this kind form a series known as the binomial distribution.

In spore germination studies, the bag of seeds might represent the total number of spores treated under similar conditions, the black seeds representing ungerminated spores and the white seeds germinated spores, while a lot of 50 would be the spores counted in a particular microscopic field. If the variations observed in spore germination tests are no greater than those found in experiments on drawing black seeds and white seeds from a bag, then the indications are that the worker has been successful in equalizing environmental conditions in the different drops. In many biological experiments, however, such an ideal is not attained and it is a matter of interest to see whether it can be attained in actual spore germination experiments.

There is a quantity called Chi-square (χ^2), which may be calculated from the data on spore germination and which served to indicate whether or not the deviations occurring in replicate experiments are due chiefly to sampling errors. The method of calculating this quantity is fully described by Fisher (4, p. 75–98), but a simple illustration may be given here. Suppose in one microscopic field 50 spores had been counted, of which 34 had germinated, while in a second field from the same material 43 had been counted of which 21 had germinated, and in a third of 52 counted 32 had germinated. The question to be answered is whether such a result is likely to be obtained through the error of sampling alone, or whether errors of technique were present also, such as a failure to maintain the three replicate tests at the same temperature. The average percentage germination is 60 in the combined tests and hence we would expect 60 per cent of 50 or 30 to germinate in the first test, 60 per cent of 43 or 26 in the second, and 60 per cent of 52 or 31 in the third. The number of spores failing to germinate in the three tests are 16, 22, and 20 respectively, while 20, 17, and 21 would be the expected values. The difference between the expected values and those actually obtained is 4 in the first case, 5 in the second, and 1 in the third. If these differences are squared and divided by each of their respective expected values, we have 16/30, 16/20, 25/26, 25/17, 1/31, and 1/21. The sum of these fractions is χ^2, and is 3.84. In order to determine whether this value would be likely to arise from errors of random sampling it is necessary to refer to a table giving the probability of occurrence of various values of χ^2. Such tables are given by Fisher (4, p. 96–97), and others. In this case the probability of a value as great or greater than 3.84 occurring by chance is between 1 in 5 and 1 in 10, and hence there is no reason to consider that the values obtained might not have arisen from variations of random sampling.

In the example given above three counts of germinating spores were made. The method may be applied, however, to any number of replicate counts, and it will be noted that the counts need not involve the same number of spores, and that actual numbers and not percentages are used

TABLE I

APPLICATION OF χ^2 TEST TO SPORE GERMINATION EXPERIMENTS

Type of experiment		Number of spores	n	χ^2	$\sqrt{2\chi^2} - \sqrt{2n-1}$	Probability of occurrence of χ^2 as great or greater than value obtained
Fungus	Treatment					
Sclerotinia americana	Control	32,004	152	129.57	−1.31	.90
"	Fungicide	50,935	199	207.44	+0.44	.33
"	Fungicide with rain test	46,518	124	196.40	+4.10	< .001
Pestalotia stellata	Control and fungicide	17,272	175	166.97	−0.41	.66
Uromyces caryophyllinus	Control and fungicide	15,864	206	270.06	+2.97	.0014
Total		162,593	856	970.44	+2.69	.0035

in calculating χ^2. In using the χ^2 table the value of n with which to enter the table is 1 in case we have duplicate observations and the spores are classified into two categories, germinated and non-germinated. If we had triplicate observations, as in the example, the value of n would be 2, that is, one less than the number of replicates. If a large number of experiments have been performed, each consisting of two or more replicate tests and values of χ^2 calculated for each experiment it is possible to examine the series as a whole by adding together the individual values of χ^2 and entering the table with n equal to the sum of the individual values of n in each experiment.

For determining the variability of spore germination tests, the results obtained during the past year on over 160,000 spores were examined by the χ^2 method. The experimental material included results on the conidia of three different species: *Sclerotinia americana* (Worm.) Nort. & Ezek., *Pestalotia stellata* B. & C., and *Uromyces caryophyllinus* (Schr.) Wint., treated with numerous toxic compounds applied as dusts and liquids. Each experiment comprised from two to ten replicate tests, and the average number of spores counted in each test was 154. Table I shows the values of χ^2 obtained in this series of experiments. When the value of n is large as in this case the probability of occurrence of a given value of χ^2 may be calculated from the fact that $\sqrt{2\chi^2} - \sqrt{2n-1}$ has an average value of zero and a standard deviation of one. Hence this expression will rarely be greater than $+2$ or less than -2 by chance. The total value of χ^2 from all the material summarized in Table I is 970.44, while the corresponding value of n is 856, and $\sqrt{2\chi^2} - \sqrt{2n-1}$ is $+2.69$.

Hence we conclude that these spore germination tests as a whole are somewhat more variable than would be expected from errors of random sampling. It is a matter of interest to determine whether the high variability is confined to certain species or certain types of experiments. If the results on *Uromyces caryophyllinus* and tests, where the dusted slides were submitted to rain before germination, are omitted, the remainder of the data gives a χ^2 value of 503.98, with a corresponding n equal to 526. The value of $\sqrt{2\chi^2}-\sqrt{2n-1}$ is -0.67, a very reasonable result. It appears then that tests on *Uromyces* and tests, where the fungicidal dust is submitted to rain before setting up, are likely to give more variable results than those using *Sclerotinia americana* or *Pestalotia stellata*. In the case of these last two species the indications are that the technique of performing the tests has been perfected, so that only sampling errors remain. A reasonable explanation of the greater variability of the rain tests is afforded by the observation that the material was not washed off the slides uniformly, but that the fungicide remaining was distributed more or less in patches, while in the case of *Uromyces*, there is a tendency for the spores to aggregate thus preventing uniform suspensions.

A further test of the agreement of the data with the results to be expected on the basis of sampling variations is obtained by tabulating the distribution of individual χ^2 values and comparing them with the expected distribution. Table II shows such a tabulation of 126 experiments (all the data except that on *Uromyces* and on the rain tests) in which the frequency of occurrence of χ^2 values having a certain degree of probability is compared with the expected frequency obtained from Fisher's table (4, p.

TABLE II

THE DISTRIBUTION OF χ^2 FOR 126 SPORE GERMINATION EXPERIMENTS COMPARED WITH THE EXPECTED DISTRIBUTION

χ^2 having a probability of	Expected frequency	Observed frequency	$\frac{(\text{Exp.}-\text{Obs.})^2}{\text{Expected}}$
>.98	2.5	3	0.10
.98–.95	3.8	4	0.01
.95–.90	6.3	9	1.16
.90–.80	12.6	15	0.46
.80–.70	12.6	13	0.01
.70–.50	25.2	19	1.53
.50–.30	25.2	21	0.70
.30–.20	12.6	17	1.54
.20–.10	12.6	14	0.16
.10–.05	6.3	4	0.84
.05–.02	3.8	5	0.38
<.02	2.5	2	0.10
Total	126.0	126	6.99

$n=11$, total $\chi^2=6.99$, therefore probability of occurrence of total χ^2 as great or greater than 6.99 is .80.

96–97). The final value of χ^2 is 6.99, while n is 11, one less than the number of classes. The probability of occurrence of such a value of χ^2 for n equal to 11 is .80. Hence we may also conclude by this method of testing that the variations are no greater than might be expected if these variations arose from random sampling.

SIGNIFICANCE OF DIFFERENCES IN SPORE GERMINATION EXPERIMENTS

In spore germination tests, as has been stated previously, we are usually interested in comparing differences in germination percentages. Since small differences will arise by chance, it is necessary to consider in any given case how large a difference must exist to enable the investigator to decide that the difference is really significant.

If it is known by application of the χ^2 test that the only errors of importance are those of random sampling, it is possible to construct a table showing the difference in percentage germination which is significant with odds of 50 to 1 for any particular number of spores used in the test (Table III). It is assumed that approximately the same number of spores is used in each of the two tests to be compared. The use of this table may be illustrated by the following typical experiment:—Two fungicides A and B were compared by means of spore germination tests, using 760 spores in one experiment, and 695 in the other. The percentage germination obtained with fungicide A was 63, while in the case of fungicide B, 56 per cent of the spores germinated. We wish to know whether the difference, 7, is significant. Entering Table III under 60 per cent and opposite 750 we see that a difference of 5.9 is significant with odds of 50 to 1. Hence it is very probable that fungicide B was more effective than fungicide A. It will be noticed that the more spores counted, the smaller the significant difference between the means of two experiments. Also the difference required to be significant depends on the germination percentage obtained. It should be emphasized that Table III only applies in case the experimental material is known to follow the binomial distribution (from the fact that a reasonable χ^2 value has been obtained), and the size of the two samples to be compared is approximately the same.

If the material is more variable than could be accounted for on the basis of random sampling errors, then a bigger difference between two tests will be necessary in order that the difference may be significant. It might still be possible to utilize Table III, by dividing the number of spores used in a test by χ^2/n, where χ^2 is the result of a large number of determinations on this material, and entering the table opposite this value instead of the number actually used (13, p. 229). For example, in the case of the rain test shown in Table I, χ^2/n is 1.58. Hence for two tests to differ significantly, using 1000 spores per test, their means should differ by 6.7, where the

TABLE III

TABLE OF DIFFERENCES NECESSARY TO GIVE ODDS OF 50 TO 1, BETWEEN THE GERMINATION PERCENTAGES OF TWO SAMPLES WITH BINOMIAL DISTRIBUTION*

Number of spores per sample	Average per cent germination of the two samples									
	1 or 99	3 97	5 95	10 90	15 85	20 80	25 75	30 70	40 60	50
10	—	—	—	—	—	41.7	45.2	47.8	51.2	52.2
12	—	—	—	—	—	38.1	41.2	43.6	46.7	47.7
15	—	—	—	—	30.4	34.1	36.9	39.1	41.8	42.7
20	—	—	—	22.2	26.3	29.5	32.0	33.8	36.2	37.0
25	—	—	—	19.8	23.6	26.4	28.6	30.3	32.4	33.1
30	—	—	—	18.1	21.5	24.1	26.1	27.7	29.6	30.2
40	—	—	—	15.7	18.6	20.9	22.6	24.0	25.6	26.1
50	—	—	10.2	14.0	16.7	18.7	20.2	21.4	22.9	23.4
60	—	—	9.3	12.8	15.2	17.1	18.5	19.6	20.9	21.3
75	—	6.5	8.3	11.5	13.6	15.3	16.5	17.5	18.7	19.1
100	—	5.6	7.2	9.9	11.8	13.2	14.3	15.2	16.2	16.5
120	—	5.1	6.6	9.1	10.8	12.1	13.1	13.8	14.8	15.1
150	—	4.6	5.9	8.1	9.5	10.8	11.7	12.4	13.2	13.5
200	—	4.0	5.1	7.0	8.3	9.4	10.1	10.7	11.5	11.7
250	2.1	3.6	4.6	6.3	7.5	8.4	9.0	9.6	10.2	10.5
300	1.9	3.3	4.2	5.7	6.8	7.6	8.3	8.7	9.3	9.5
400	1.6	2.8	3.6	5.0	5.9	6.6	7.2	7.6	8.1	8.3
500	1.5	2.5	3.2	4.4	5.3	5.9	6.4	6.8	7.2	7.4
600	1.3	2.3	2.9	4.0	4.8	5.4	5.8	6.2	6.6	6.7
750	1.2	2.1	2.6	3.6	4.3	4.8	5.2	5.5	5.9	6.0
1,000	1.0	1.8	2.3	3.1	3.7	4.2	4.5	4.8	5.1	5.2
1,500	0.8	1.5	1.9	2.6	3.0	3.4	3.7	3.9	4.2	4.3
2,000	0.7	1.3	1.6	2.2	2.6	3.0	3.2	3.4	3.6	3.7
2,500	0.7	1.1	1.4	2.0	2.4	2.6	2.9	3.0	3.2	3.3
3,000	0.6	1.0	1.3	1.8	2.2	2.4	2.6	2.8	3.0	3.0
4,000	0.5	0.9	1.1	1.6	1.9	2.1	2.3	2.4	2.6	2.6
5,000	0.5	0.8	1.0	1.4	1.7	1.9	2.0	2.1	2.3	2.3
6,000	0.4	0.7	0.9	1.3	1.5	1.7	1.9	2.0	2.1	2.1
7,500	0.4	0.7	0.8	1.2	1.4	1.5	1.7	1.8	1.9	1.9
10,000	0.3	0.6	0.7	1.0	1.2	1.3	1.4	1.5	1.6	1.7

* The use of this table requires that the material follow the binomial series as shown by the χ^2 test. For a modified application under certain conditions see page 239.

germination is about 50 per cent, instead of 5.2 as would be the case if the only variations were those of random sampling.

TOXICITY CURVES

When a series of spore germination tests is performed in the presence of a toxic agent whose concentration increases from test to test, the percentage germination may be plotted against concentration giving a toxicity curve which is usually S-shaped. A similar curve is obtained if the percentage germination after varying periods of exposure to the toxic

agent is plotted against the time of exposure, with a fixed concentration of the toxic agent. Either of these two methods may be used to compare the effect of different fungicides. The authors have preferred to employ the first method, since in this case it is not necessary to remove the toxic agent by washing or centrifuging with possible injury to the spores. The toxicity curve is not S-shaped in all cases; sometimes a J-shaped curve is obtained. There has been much discussion regarding the causes giving rise to toxicity curves of a particular shape. Smith (9, 10) has shown that both

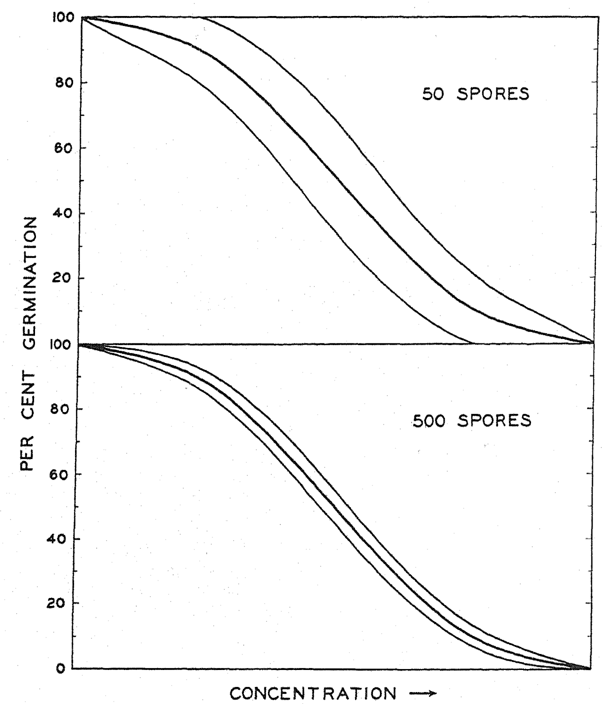

Figure 1. Typical spore germination toxicity curves. The outside curves represent the range covered by 3.46 times the probable error, which corresponds to odds of 50 to 1. In the upper diagram each point on the curve is obtained from 50 spores, and in the lower from 500 spores.

types of curves may be plausibly explained by the individual differences in resistance among the spores used in a test. Recently this subject has been reviewed by Rahn (7, 8) who offers an explanation based on the number of molecules per cell which must react with the toxic agent to cause death, in the case of bacteria. In the experiments performed by the present authors, individual differences in resistance must be an important factor, since even in control experiments with no added toxic agent, it is not often that 100 per cent germination is obtained.

Two typical toxicity curves are shown in Figure 1. The limits representing 3.46 times the probable error are indicated by the bounding lines in the figures and it may be seen how much the precision increases when 500 spores are used to determine each point instead of 50 spores. Approximately 98 per cent of the determinations will give results lying within these lines. These lines trace out a "zone of uncertainty," and it is obvious that when two different toxic agents are compared, these zones must not overlap to any great extent if we are to conclude that the two substances are really different in their effect. The steepest portion of the curve is not far from a germination percentage of 50, and it is in this region that failure to control environmental conditions adequately will lead to the greatest variation in germination percentage between replicate tests. It is also in this region that we can distinguish most precisely between two slightly different concentrations of the toxic agent by means of spore germination if the other environmental factors are adequately controlled. If causes of variation other than those of random sampling are present, then the "zone of uncertainty" would be wider than shown in the figure. The number of spores or other individuals to be employed in the determination of a toxicity curve, must be governed by the accuracy desired, the ease of obtaining material, and the variability of the particular material under consideration. A further discussion of toxicity curves and the factors to be considered for obtaining accurate results are to be found in papers by Trevan (11) and by Durham *et al.* (3).

SUMMARY

1. The necessity of accurate spore germination tests has been emphasized in relation to the evaluation of fungicides in the laboratory, as well as in life history studies, each of which constitute an important phase of plant pathological work.

2. The variations encountered in replicate tests fall into two categories, those due to a lack of uniformity in the conditions governing germination and those due to "errors of random sampling." The former may be reduced to a negligible factor by careful technique, while the latter are unavoidable, and quite definite in magnitude. It is possible by means of a simple mathematical test (the χ^2 test) to show to what extent errors of technique have been eliminated.

3. The χ^2 test has been applied to results on three species, totalling 160,000 spores germinated in the presence of various fungicides. It was found that in the majority of experiments which involved spores of *Sclerotinia americana* and *Pestalotia stellata* the errors due to faulty technique were negligible. In the case of tests where the fungicide was subjected to artificial rain, before germination of the spores, and tests using spores of *Uromyces caryophyllinus*, the results were more variable.

4. A table is presented which gives the differences between the germination percentages of two samples with binomial distribution, necessary to give odds of 50 to 1 that the difference is significant.

5. The form of toxicity curves and the theories underlying them are discussed and two typical curves are illustrated, showing the deviations to be expected from the errors of random sampling.

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VALUE OF PEATS FOR MINERAL SOIL IMPROVEMENT

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Because of the scarcity of manures there is an increasing interest in the use of domestic peats as a source of organic matter for soil improvement. While in general farming green manures usually are a cheaper source of organic matter, in intensive production and in the growing of ornamentals green manure can not be used. Some new source of organic matter should be developed to fill this need. A review of the available literature on the use of peats as an organic source shows that the information is fragmentary. Generally the peats used in the investigations are not classified; also often the plants from which the peats were derived and the horizon of the deposit from which the samples were taken, are not given. This paper deals with certain physical and chemical characters of 13 different peats, determines their value for plant production when variously treated, and studies the rate of nitrate formation in several of them.

REVIEW OF LITERATURE

Dreiman (2) applied about 145 cubic yards of peat per acre to a sandy soil and nearly doubled the yield of barley. Hall (3) reports ammonification to take place rapidly in peat formed from reed and aquatic peats of the southwestern Transvaal, and also crops, when grown in them, respond strikingly to applications of superphosphate. Johnstone (4) reports in favor of adding peat to heavy Essex soils. He suggests the use of the peat in the moist condition. For strawberry runners and the flower garden one-third peat should be used with the top four inches of soil. Where used on a large scale, as much as five tons per acre should be applied along with dung and fertilizer salts. Kreuger (5) found upon the addition of large amounts of peat (300 cubic meters per hectare) to sandy soils, the yield of oat straw to be increased, but the yield of grain to be decreased. The best results were obtained when there was an abundance of water present. The studies included pot and field methods. Langley (6) discusses the various uses of peat in plant production. The acid type is valuable for the propagation of cuttings when used with equal parts of sand. The less acid ones are valuable sources of organic matter being satisfactory substitutes for leaf-mold and manure. They are valuable for growing bulbs and potted plants, for storage media, gardens, mulches, in flower and shrub gardens, and for lawn establishment and improvement. Laurie (7) demonstrated with marigold, petunia, zinnia, verbena, cosmos, chrysanthemum, snapdragon, balsam, hydrangea, geranium, lettuce, and tomato the superiority of sedge peat over sphagnum peat in the greenhouse. He found it to be a satisfactory substitute for leaf-mold and for the greater part, at least, of the manure employed. A very satisfactory mixture resulted from the addition of

one part by volume of manure to 20 of the peat. McCool and Wheeting (8) studied the relative activities of peats taken from several deposits. They found wide differences in the rate at which soluble salts are formed in peats. The most active materials were those taken from the surface of the deposits. Valmari (9) found the largest amount of ammonia to be produced in peats with 70 to 80 per cent of their total water-retaining power. The optimum amount for nitrate formation was about 10 per cent lower than that for ammonification. The presence of basic materials was advantageous for the nitrate formation. The addition of nitrate of soda and ammonium sulphate at first decreased the amount of soluble nitrogen but later on increased the amount of it. The addition of potash and phosphate to the peats aided in nitrogen transformation in them. Willis (10) incubated a muck, having 52.8 per cent volatile matter, pH 3.62, with and without lime. The nitrates in the unlimed portion increased from 28 parts to 114 parts per million. Where lime, either as the hydrate, limestone, or marl, was added to it, the nitrates increased on the average to 205 parts per million of the moisture-free muck. Wilson and Townsend (11) found nitrate nitrogen to accumulate rather rapidly in a newly cleared muck, and to a somewhat less extent in one that had been under cultivation 20 or more years. The rate of nitrification was greatly reduced by the addition of Timothy and clover hay respectively. The retarding action of the former appeared to endure longer than the latter.

METHODS AND MATERIALS

The mineral matter content of the peats was determined by burning five-gram oven-dried samples in an electric furnace heated to 700° C. The total nitrogen was determined in the usual manner in the entire samples as well as in the material which remained in suspension in water 60 minutes. The latter was obtained by dispersing with the Bouyoucos' (1) apparatus. The dispersed samples were placed in 1000 cc. cylinders, distilled water added, and the contents shaken. After 60 minutes the suspension was removed and the process repeated until the liquid was clear upon standing the allotted time. The material was then caught on a filter in a Buckner funnel. The samples in which the total nitrogen was determined, were dispersed and treated with the same amount of distilled water which was required to remove the fine material.

The pH values were determined by means of the quinhydrone electrode. Seventy-five cc. of distilled water were added to 25 grams of the peats, the mixture stirred and the readings taken 18 hours later.

In determining the rate of nitrate formation 200-gram samples of the moist peats, which had been leached to reduce the nitrate content, were inoculated with 5 cc. of a fertile soil suspension. This was made up by shaking 50 grams of fertile garden soil with 100 cc. of distilled water. The 500 cc.

Erlenmeyer flasks in which the peats were placed were loosely plugged with cotton and incubated at temperatures ranging from 22° to 24° C.

SOURCES OF PEATS

Several hundred pounds were taken from each of several deposits in central Michigan by R. L. Cook of the Michigan Agricultural Experiment Station. These were shipped in barrels to the Boyce Thompson Institute. Samples were taken from certain deposits within a radius of about 80 miles from Yonkers, New York. With few exceptions the peats were identified by Dr. A. P. Dachnowski-Stokes of the United States Department of Agriculture, Bureau of Chemistry and Soils. His classification and the type names used in the Michigan Soil Survey are given in Table I.

TABLE I

Classification of Peats

Group No.	Description	Laboratory No.	Type and horizon
1	Woody reed and sedge decomposition well advanced	4	Carlile A
2	Reed and sedge decomposition moderately advanced	5 6 11 12	Carlile B Carlile C Houghton A Houghton B
3	Woody with admixture of sedge and reed largely decomposed	8	Rifle A
4	Reed and hypnum	9	Rifle B
5	Reed partly decomposed, with very small amounts of plant remains from sphagnum moss	10	Greenwood B
6	Sedge—reed containing crystals of calcium sulphate and other salts	7	Kirston B
7	Sedimentary with diatoms	20	Undetermined C
8	Sandy and silty organic debris; chiefly sedimentary material with small amounts of wood fragments	1 2 3	Undetermined: Surface Undetermined: 4 ft. from surface Undetermined: 8 ft. from surface
9	Wood fibrous sedge with admixture of woody material and charred woody fragments	13	Undetermined 0 to 12 in. from surface
10	Sedimentary fibrous sedge partly decomposed	14 17	Undetermined 6 ft. from surface
11	Woody fibrous material, undetermined origin	16	Undetermined 4 to 12 in. from surface
12	Dark brown in color, very acid, very sticky, undetermined origin	18 19	4 to 12 in. from surface 4 ft. from surface
13	Sphagnum partly decomposed	15	Undetermined

RESULTS

ANALYSES OF PEATS

According to the data in Table II, rather wide differences in the residue obtained upon ignition of the peats were obtained. The surface material from a given deposit contained more than did that taken at greater depths from the surface. Peats 1, 2, 3, 7, and 8 contained comparatively small amounts of organic matter, an unfavorable condition, since peat is purchased and utilized primarily because of its organic matter content. The material obtained from 6 of 13 peats, which remained in suspension in a 1000 cc. cylinder 60 minutes, contained a higher percentage of nitrogen than did the entire samples. It is probable that the material which would remain in suspension longer periods of time would contain more nitrogen than did that which was studied, because there is a tendency for the nitrogen content to increase as the plants making up the less acid peats decompose. Then too the leaves of plants, as a general rule, contain more nitrogen than do the stems. The peats may be thrown into three groups with respect to their pH values, the very acid peats or Nos. 10, 15, 18, and 19, the medium acid peats or Nos. 1, 2, 3, 16, and 17, and the slightly acid peats or the remainder of them.

TABLE II
ANALYSES OF PEATS

Peat No.	Per cent residue upon ignition	N per cent fine material, 60 minutes sedimentation	N per cent entire sample	pH
1	53.75	2.17	3.27	5.50
2	41.65	2.93	1.63	5.46
3	40.25	2.54	2.14	5.04
4	21.31	3.05	3.26	6.27
5	14.73	2.93	3.22	6.14
6	11.67	2.13	2.63	6.12
7	34.66	——	2.68	6.25
8	30.97	2.04	2.57	6.24
9	11.27	3.01	2.03	6.35
10	3.33	——	3.10	3.48
11	13.52	3.93	3.35	6.75
12	12.31	3.15	2.62	6.30
13	9.70	——	3.02	5.22
14	6.38	2.81	2.95	6.85
15	3.45	——	0.75	3.39
16	17.60	2.37	1.78	5.31
17	14.30	3.01	——	5.61
18	9.79	——	1.36	3.40
19	7.45	——	1.24	3.48
20	12.10	——	2.98	6.24

FORMATION OF NITRATES IN PEATS

It is apparent from the results presented in Table III, that considerable of the nitrogen in peats is readily nitrified. The woody reed and sedge

TABLE III
NITRATE FORMATION IN PEATS

Peat No.	Increase in NO_3 p.p.m. of dry peat	
	18 days	28 days
4	994	1240
5	520	750
6	109	154
7	645	835
8	345	462
9	115	345
11	222	426
12	390	546
14	194	465

peat No. 4, the reed and sedge peat No. 5, and the sedge-reed peat No. 7, were especially active in the formation of nitrates. It may be that the removal of much of the soluble organic matter by leaching in the preparation of the samples reduced their activity somewhat. These results are not surprising when cognizance is taken of the fertilizer practices followed in the production of crops on the organic soils. Usually nitrogen is not applied for the production of other than the rapidly growing and heavy yielding crops such as onions, mint, celery, and cabbage.

FERTILIZER REQUIREMENTS OF PEATS

The fertilizer and lime requirements of several peats as measured by the growth of rye grass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) have been determined. One-gallon glazed jars provided with drainage outlets at the bottom, were employed as the containers. They were placed in the open on frames about one inch from the surface of the ground. The fertilizer salts consisted of a solution composed of 0.03 g. $NaNO_3$, 0.04 g. $Ca(H_2PO_4)_2$, 0.04 g. KCl and were mixed with the top three inches of soil. Where lime was added it was mixed throughout the contents of the jars. The same amount of rye grass seed was sown in each container. The data presented in Table IV are the average green weights of the grass obtained from three cultures.

According to the data presented in Table IV and illustrated by Figure 1, A there was considerable growth of grass in all of the peats without treatment except the very acid ones. The addition of lime to the latter, however, resulted in yields which compared favorably with the others. The peats taken from the surface of the deposits or Nos. 1, 4, and 11 were more productive than those removed from greater depths or peats 2, 3, 5, 6, and 12. Grass seeded in fertilized peats 9 and 10 failed when taken from the deposits. Later on the factor or factors retarding the growth of the grass largely disappeared (Fig. 1, B). Peats 2, 7, and 9 did not need the potash

TABLE IV

GROWTH OF RYE GRASS IN PEATS; DURATION OF GROWTH 28 DAYS

Peat No.	Treatments				
	Check	K	P.K.	N.P.K.	N.P.K. Ca
	g.	g.	g.	g.	g.
1	11.5	16.0	17.0	20.6	18.2
2	10.7	10.7	18.7	20.9	—
4	27.0	—	32.5	43.6	—
5	10.3	12.7	14.6	21.4	—
6	9.1	10.5	12.4	20.6	—
7	17.1	14.6	22.1	26.0	—
8	4.1	4.7	—	8.7	16.4
9	6.3	7.1	14.2	20.6	—
10	0.8	7.1*	7.7*	—	17.5
11	14.3	23.0	25.8	31.9	32.0
12	10.7	18.6	16.8	39.8	31.2
14	9.5	19.6	16.8	22.0	22.8
18	2.9	—	—	—	23.4
	11.7*	14.7*	15.8*	—	—
19	3.6	—	—	—	25.6
	12.3*	16.7*	12.9*	—	—
20	9.2	13.5	13.3	19.6	16.9

* 2 g. $CaCO_3$ added.

TABLE V

GROWTH OF WHITE CLOVER IN PEATS; PERIOD OF GROWTH 63 DAYS

Peat	Treatment		
	Check	K	P.K.
	Av. green wt. in g.	Av. green wt. in g.	Av. green wt. in g.
1	12.3	12.9	16.6*
2	14.5	14.6	32.5
4	19.8	35.7	43.1
5	12.1	9.8	39.6
6	1.5	1.4	14.8
7	17.2	14.3	34.5
8	4.5	5.1	32.1
9	0.5	1.3	50.5
10	0.0	—	—
	4.5*	10.2*	36.8*
11	13.5	23.6	36.5
12	3.1	16.2	38.9
14	6.8	2.5	41.4
18	0.0	—	—
	1.6*	1.1*	29.7*
19	0.0	—	—
	0.0*	2.0*	21.3*
20	0.8	1.5	32.3

* 2 g. $CaCO_3$ added.

application for the growth of the grass, but an addition of the phosphate was beneficial. With the exception of peats 1 and 2 the productivity of the peats was greatly increased by the nitrogen added to them.

The turf and grass roots were removed from certain of the jars, the contents refertilized with phosphate and potash, and inoculated white clover seed sown in them. Unlike the rye grass this plant is very sensitive

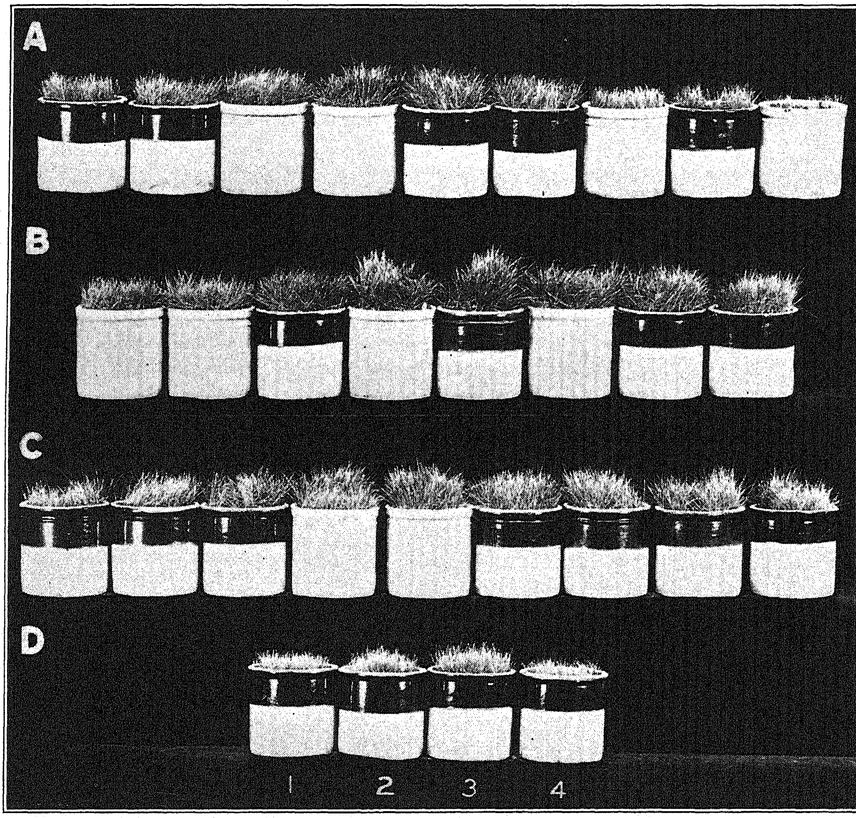

Figure 1. A. Rye grass growing in untreated peats. Left to right: Peat Nos. 6, 5, 7, 4, 11, 12, 9, 8, 10. B. Rye grass growing in fertilized peats. Left to right: Peat Nos. 9, 10, 11, 4, 12, 17, 5, 6. C. Rye grass growing in mixtures of one part of peat and two parts sandy loam plus fertilizer salts. Left to right: Peat Nos. 10, 11, 8, 7, 4, 5, 6, 12, 9. D. Rye grass growing in sandy loam. (1) Phosphate and potash; (2) Nitrate, phosphate, and potash; (3) Nitrate, phosphate, potash, and limestone; (4) No treatment.

to a deficiency of phosphorus. As may be ascertained from the data in Table V, the clover was a failure in the untreated peats 6, 8, 9, 10, 12, 14, 18, 19, and 20. The addition of potash to peats 10, 11, and 12 was beneficial and strikingly so in the case of peat 4. Increases in the yields in all cultures resulted from the use of the phosphate along with the potash. Lime also was essential for the success of this plant in the very acid peats. Another

set of cultures was made up in which nitrate of soda and potash were added. The results obtained were similar to those derived from the above cultures which did not receive the phosphate.

The nodules on the roots of the clover plants were small and few in number, where the phosphate was omitted from all peats except 4.

PEATS AS MINERAL SOIL CONDITIONERS

Several of the moist peats were employed in studies on their value as mineral soil improvers. An infertile sandy loam surface soil (pH 5.4) was employed as the basic material. In the first set of experiments two parts by volume of the sandy loam and one part by volume of the peat were mixed together. Some cultures received fertilizers salts and lime while others did not. Rye grass was seeded and grown under conditions the same as in the previous series. The results obtained are summarized in Table VI

TABLE VI

GROWTH OF RYE GRASS IN PEATS AND SANDY LOAM MIXTURES; PEAT TO SOIL RATIO BY VOLUME 1 TO 2; PERIOD OF GROWTH 26 DAYS

Peat	Treatment			
	Check	P.K.	N.P.K.	N.P.K. Ca
	Av. green wt. in g.	Av. green wt. in g.	Av. green wt. in g.	Av. green wt. in g.
1	11.7	10.5	9.2	19.8
2	12.5	13.4	17.5	21.4
4	20.4	19.4	28.7	—
6	6.7	—	14.4	—
7	14.8	14.1	20.0	—
8	10.0	13.8	13.9	—
9	8.7	8.7	15.2	—
10	9.4	7.3	—	9.5
11	13.1	15.4	15.6	—
12	13.5	16.2	18.5	—
14	9.7	11.2	17.5	14.2
Sandy loam	2.7	3.4	7.4	9.3

and illustrated by Figure 1, C and D. Reference to the yields obtained from the untreated peats (Table V) brings out that diluting them with the sandy loam was beneficial to peats 2, 8, 9, 10, and 12 but lowered the efficiency of peats 4, 6, and 7. In all cases the presence of the peats improved the sandy loam soil markedly. The application of the phosphate and potash without nitrogen was not effective. Where nitrogen was used along with them, the yields of the grass increased. The best results were obtained from the containers which carried peats 4, 2, and 7.

An additional series of experiments was run. The mineral soil employed was the B horizon or the subsurface layer of the sandy loam, the pH of which was 5.40. The peat to soil ratio was varied but the fertilizer treat-

TABLE VII

GROWTH OF RYE GRASS IN PEAT AND SANDY LOAM, B HORIZON MIXTURES; PERIOD OF GROWTH 27 DAYS

Peat	Peat to soil ratio by volume				
	1–1	1–2	1–3	1–4	0–1
	Av. green wt. in g.	Av. green wt. in g.	Av. green wt. in g.	Av. green wt. in g.	Av. green wt. in g.
14	17.6	17.8	16.0	11.5	11.6
6	20.6	20.8	18.1	17.1	—
2	22.8	26.0	20.5	13.1	—

ment was the same throughout or 2 grams of a 4–8–6 commercial fertilizer. According to the data in Table VII the yields of the grass in the cultures in which were present peats 14 and 6 did not decline markedly until the peat to subsoil ratio reached 1 to 4. The best ratio for peat No. 2, the sandy loam soil, was 1 to 2.

The remaining tests were conducted in the greenhouse. Peat 14 was mixed with an unweathered coarse sand (pH 6.70) and also the sandy loam B horizon. Finally, sandy loam, as in the first set of experiments, was employed. Rye grass was the plant indicator used for the sand and subsoil mixtures. Rye grass (*Lolium perenne* L.), nasturtium (*Tropaeolum majus* L.), and tobacco (*Nicotiana tabacum* L.) were grown in the peat-sandy loam

TABLE VIII

VALUE OF FIBROUS SEDGE PEAT AS A MINERAL SOIL IMPROVER

Soil	Plant used	Peat to soil ratio				
		1–1	1–2	1–3	1–4	0–1
		Av. green wt. in g.	Av. green wt. in g.	Av. green wt. in g.	Av. green wt. in g.	Av. green wt. in g.
Sand	Rye grass	35.5	23.1	21.6	—	16.7
B horizon + 2 g. 4–8–6 fertilizer	Rye grass	17.6	18.1	16.9	11.5	0.75* 8.5
Sandy loam + 6 g. 4–8–6 fertilizer	Nasturtium	—	145.0	142.0	124.0	54.9* 95.2
Sandy loam + 6 g. 4–8–6 fertilizer	Tobacco	246.3	250.4	212.1	—	35.8* 125.2

* No fertilizer.

mixtures. The results obtained are summarized in Table VIII and illustrated by Figure 2. The rye grass made the largest growth where the ratio of peat to sand was 1 to 1, and the tobacco plants did best in the peat-sandy loam ratio of 1 to 2. The growth of nasturtium was decidedly less vigorous in the jars containing 1 part of peat and 4 parts of sandy loam, than it was in those in which the ratios were narrower. It would seem from these results that a satisfactory soil may be made up by using a good grade of peat,

FIGURE 2. Tobacco growing in soil and peat mixtures. (1) Sandy loam soil no treatment; (2) Sandy loam soil+fertilizer salts; (3) Peat no treatment; (4) 1 part of peat+3 parts of sandy loam+fertilizer salts; (5) 1 part peat+4 parts sandy loam+fertilizer salts.

fertilizers, and either a poor surface soil, sand, or subsoil. If the peat or mineral soil is too acid, lime should be included in the treatment.

SUMMARY

Widely different classes of peat were employed in these studies. The mineral matter content ranged from 3.45 to 53.75 per cent. The maximum amount of nitrogen was 3.35 and the minimum was 1.78 per cent. The material obtained from 7 of 13 peats, which remained in suspension in a 1000 cc. cylinder 60 minutes, contained more nitrogen than did the entire samples.

The nitrate formation in nine peats was determined after 18 and 30 days respectively. Contrary to popular conception these peats were not

inert in this respect. Although the amount of nitrates formed varied somewhat, the differences were not so great as might be predicted when the differences in the origin of the peats are considered. When cognizance is taken of the successful fertilizer practices on peat lands, however, these results are not surprising.

Although considerable growth of rye grass took place in all the peats except the very acid ones, the maximum yields were obtained from applications of fertilizer salts carrying nitrogen, phosphorus, and potassium. The very acid ones did not respond to these until lime had been applied.

Unlike rye grass, white clover failed to give satisfactory results, with one exception, unless phosphorus was added to the peats. Examination of the roots of the clover plants grown in the phosphorus-deficient peats revealed the nodules to be comparatively small in size and fewer in number than were those on the roots of the plants produced in the phosphorus-treated peats.

Unproductive surface sandy loam soil, sandy loam subsoil, and sand subsoil proved to be satisfactory media for the growth of rye grass, white clover, tobacco, and nasturtiums, when peats and fertilizer salts were added to them. The best ratio of peat to mineral soil was narrower for sand than it was for the finer textured soils employed.

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USE OF PEATS IN COMPOSTS TO INCREASE NITRIFICATION AND PLANT GROWTH

M. M. McCool

Peats, owing to their high organic matter and physical properties, are looked upon as valuable substitutes for manure and other forms of organic matter in soil improvement. There is a growing interest in the possibility of increasing their efficiency by composting. Although there are reports which show certain peats may be made more valuable by composting with suitable materials, there is need for much additional study, especially a comparison of the value of different kinds of peat when composted.

In the work reported in this paper several peats described in a previous publication (4) have been composted with different combinations of fertilizer salts, lime, straw, horse manure, and mineral soil. The nitrate present in them has been determined. In addition the comparative value of the composts and commercial manures such as dried cow manure, sheep manure, stockyard manure, and "driconure" for the production of certain plants has been studied.

REVIEW OF LITERATURE

Collison and Conn (1) hastened the decomposition of straw by mixing with it wet peat or muck, lime, and fertilizer salts. Six hundred pounds of the muck fresh from the deposit, nearly saturated with water, and 500 pounds of air-dry wheat straw were mixed and composted 11 months in the open before their fertilizer value was determined.

Jakuschkin (2) employed a peat which contained 2.98 per cent nitrogen in his investigations on the formation of nitrates from the nitrogen compounds. The peat was crushed and placed in pots 15 cm. deep. The moisture content of the peat was maintained at 75 per cent. Calcium carbonate, $NaCl$, KH_2PO_4, and several grams of cultivated soil were added to each of the pots. The most rapid formation of nitrate nitrogen took place during the first 30 days of the incubation period. At the close of the experiment there were 1375 mg. of nitrogen per kilogram of air-dry compost. Where 120 mg. of $(NH_4)_2SO_4$ were added to the compost, the nitrate nitrogen increased somewhat more rapidly and to a greater extent than it did in those which had not received this treatment.

In another set of experiments *Bacillus mycoides* was added to the composts and the nitrate likewise determined. The addition of this organism resulted in a small increase in the nitrate content above that found in the compost which had received the cultivated soil.

Kupryanov and Rozonov (3) found composts of sphagnum peat and phosphorite to carry 20 to 30 times as much water-soluble phosphate as did similar composts made with wood sedge. It represented 32 per cent

of the total phosphorus content of the phosphate added. Manure made with peat litter was more rapidly nitrified in the soil than that made with straw as a litter. In the field trials the former was markedly superior to the latter in the production of potatoes and rye. Composts made of sedge peat and wood ashes increased the field rye 26 per cent above the control or untreated soil.

Manns and Goheen (5) added inoculated soil, plant ash, carbohydrate, K_2CO_3, superphosphate, $CaCO_3$, Thomas slag, cyanamid, and guano to muck in making up an elaborate series of experiments. Four-gallon jars were employed as the containers. The muck when treated properly became a very favorable medium for a beneficial flora. Rye and millet grew well in certain of the mixtures without the addition of nitrogen. They did not find any correlation between the nitrate content of the compost and the yield of rye and millet grown in them.

PEAT COMPOSTS

A partly decomposed, sedimentary, fibrous, sedge peat (4) was used in making up the A series of composts. Wooden containers, one cubic yard capacity, were filled with the peat and various materials as given in Table I. The peat contained 70 per cent moisture. The horse manure was passed

TABLE I

NITRATE FORMATION IN PEAT COMPOSTS. SERIES A. STARTED AUGUST 3, 1931

Compost No.	Treatment per cubic yard moist peat						NO_3 p.p.m. dry compost Mar. 8 1932
	Ammonium sulphate, lbs.	Phosphate, lbs.	Potash, lbs.	Manure, lbs.	Lime, lbs.	Garden soil, lbs.	
1	0	0	0	0	0	0	933
2	7.5	15	5	0	50	0	4124
3	7.5	15	5	20	50	0	5130
4	7.5	15	5	20	25	0	5715
5	7.5	15	5	20	10	0	5814
6	7.5	15	5	20	0	0	5181
7	7.5	15	5	*	50	0	4651
8	**	15	5	20	50	0	4651
9	7.5	15	5	5	25	0	5750
10	7.5	15	5	3 bu.	10	0	5882
11	7.5	15	5	0	10	20	5155

* Manure extract from 20 lb. manure.
**Nitrogen as urea.

through a one-fourth inch screen before it was incorporated with the peat. The lime employed was a finely ground dolomitic stone, and the superphosphate contained 16 per cent P_2O_5.

Compost series B consisted of rye straw, the above peat, horse manure, fertilizer salts, and lime. Ten-gallon glazed jars served as the containers.

The temperature of the room in which they were stored ranged from 20° to 25° C. The rye straw was made up to 70 per cent moisture before it was used. The contents of container number one weighed 50 pounds and all others 25 pounds. Unless otherwise stated the lime and salt treatment consisted of 170 grams of ammonium sulphate, 340 grams of superphosphate, 121 grams of potassium sulphate, and 264 grams of limestone. One pound of horse manure was added. The manure extract was obtained from one pound of the manure. The make-up of the composts is presented in Table II.

TABLE II

Nitrate Formation in Peat Composts. Series B. Started August 7, 1931

Compost No.	Treatment of fibrous, sedimentary, sedge peat	NO_3 p.p.m. dry compost Mar. 9, 1932
1	No treatment	933
2	5 parts by weight peat + 1 part straw, manure, salts, limestone*	7205
3	1 " " " " + 1 " " " " "	8740
4	1 " " " " + 1 " " " " "	8120
5	1 " " " " + 1 " " manure extract**, salts, and limestone	8070
6	1 part by weight peat + 1 part straw, manure, salts, 2/5 limestone	8539
7	1 " " " " + 1 " " " " o "	5870
8	1 " " " " + 1 " " " 1/2 " 2/5 "	5835
9	1 " " " " + 1 " " salts, 2/5 limestone	4307
10	1 " " " " + 1 " " no treatment	15
11	Straw + manure, salts, 2/5 limestone	0

* 170 g. $(NH_4)_2SO_4$ + 340 g. superphosphate + 121 g. K_2SO_4 + 1 lb. horse manure + 264 g. limestone.

** Manure extract from 1 lb. manure.

An additional lot of composts, series C, was made up from different peats (4) placed in four-gallon jars, and incubated under the same conditions as in the previous series. The fertilizer and lime mixture consisted of seven pounds of ammonium sulphate, seven pounds of superphosphate, three and one-half pounds of potassium sulphate, and eight pounds of limestone. Unless otherwise stated each gram of peat and mixture received 0.02 gram of this combination and 0.02 gram of manure. These composts are given in Table III.

Four-gallon jars were filled with the composts comprising series D. In this series the ratio of manure to peat varied widely. The fertilizer salt and lime treatment consisted of 0.02 grams of a mixture composed of one part $(NH_4)_2SO_4$, one part superphosphate, one-half part K_2SO_4, and one part limestone, per gram of compost. The make-up of the composts is presented in Table IV.

NITRATE NITROGEN IN PEAT COMPOSTS

The nitrate nitrogen in the various composts was determined after they had been thoroughly mixed. Twenty-five grams, dry basis, of each were

TABLE III

NITRATE FORMATION IN PEAT COMPOSTS. SERIES C. STARTED SEPTEMBER 21, 1931

Compost No.	Treatment of peats		NO_3 p.p.m. dry compost Mar. 10, 1932
1	2 parts wet peat No. 1+1 part wet straw*		556
2	2 " " " " 2+1 " " "		262
3	2 " " " " 3+1 " " "		276
5	1 " " " " 14+1 " " "		310
6	1 " " " " 14+2 " " "		354
7	2 " " " " 14+1 " " "		8798
8	4 " " " " 14+1 " " "		8590
9	2 " " " " 14+1 " " "		7152
10	2 " " " " 14+1 " " "	manure 1–25	7779
11	2 " " " " 14+1 " " "	manure 1–50	7444
12	2 " " " " 14+1 " " "	liquid manure	5049
13	2 " " " " 9+1 " " "		6107
14	2 " " " " 15+1 " " "		879
15	2 " " " " 12+1 " " "		7636
16	2 " " " " 4+1 " " "		9667
17	2 " " " " 11+1 " " "		6383
18	2 " " " " 6+1 " " "		125
19	2 " " " " 5+1 " " "		4743
20	2 " " " " 8+1 " " "		3921

* Plus 0.02 g. of a mixture consisting of 1 part (by weight) of $(NH_4)_2SO_4$, 1 part superphosphate, 1.1 part limestone, and 0.5 part of K_2SO_4 per g. of peat and straw mixture, and 1 g. manure to 1 g. of peat and straw mixture.

TABLE IV

FORMATION OF NITRATES IN FIBROUS SEDGE PEAT COMPOSTS. SERIES D. STARTED OCTOBER 3, 1931

Compost No.	Treatment of peat	NO_3 p.p.m. dry compost Mar. 15, 1932
1	1 part manure+1 part wet peat + salts + lime*	7129
2	1 " " +3 " " " + " + "	8865
3	1 " " +3 " " "	—
4	1 " " +5 " " " + salts + lime	6580
5	1 " " +10 " " " + " + "	8570
6	1 " " +1 " mixture**+ " + "	1333
7	1 " " +5 " " + " + "	1203
8	1 " " +12 " " + " + "	380
11	1 " " +40 " peat No. 14 +salts+lime	7155

* 0.02 g. of a mixture composed of 1 part $(NH_4)_2SO_4$, 1 part superphosphate, 1/2 part K_2SO_4, and 1 part limestone per g.
** Mixture = 2 parts peat+1 part straw.

placed in the dispersing machine together with 250 cc. of N/500 copper sulphate and dispersed ten minutes. Previous studies on the leaching of nitrates and other soluble salts from peats showed that their removal in the usual manner without dispersing required much leaching. The phenol disulphuric acid method was used in determining nitrates in the solutions. The results obtained are given in Tables I, II, III, and IV.

The nitrate content of the composts in series A varied somewhat but not so much as might be predicted. The untreated peat contained 933 parts per million of nitrates and they ranged in amount from 4124 parts per million in compost 2, where no manure was added, to 5882 parts per million in compost 10, which contained three bushels of manure. It was apparent also that a small amount of manure, five pounds per cubic yard, as in compost 9, served as well as much larger additions. The presence of ten pounds of limestone with the fertilizer salts and manure was slightly more advantageous in the production of nitrates than either larger amounts or its omission. Nitrogen in the form of urea was only slightly less efficient than nitrogen in the form of ammonium sulphate as illustrated by a comparison of the amount of nitrates in compost 8 with those in compost 3.

The nitrate content of the composts of series B varied widely. The addition of the manure extract to compost 5 resulted in the presence of about as much nitrates as did manure from which the extract was made. The composts from which the lime was omitted and the fertilizer salts reduced, contained less nitrates than did thosewhich received the full quota of these. There were only 15 parts per million of nitrates in compost 10, which was made up of one part of peat and one part of straw. No nitrates were detected in compost 11 which contained no peat.

It is obvious from the data in Table III that peats when composted with straw, manure, fertilizer salts, and lime vary greatly in the amount of nitrates accumulated at the end of six months. The most active in this respect were the well decomposed woody reed and sedge peat number 4, the partly decomposed sedimentary, fibrous, sedge peat number 14, the moderately decomposed reed and sedge peats numbers 12, 11, and 5, and the reed and hypnum peat number 9. The sandy and silty organic sedimentary debris with small amounts of wood, or numbers 1, 2, and 3, and the partly decomposed sphagnum peat number 15 were sluggish so far as the accumulation of nitrates were concerned.

The most satisfactory ratio of peat number 14 to straw was 2 to 1. The presence of larger amounts of straw in the mixture resulted in less than 400 parts per million of nitrates. The omission of manure from the mixture of peat and straw, or compost number 9, resulted in the accumulation of somewhat less nitrates than resulted from its presence.

Although the manure to peat ratio in composts 1, 2, 4, 5, and 11, series D, Table IV, ranged from 1 to 1 to 1 to 40, the amount of nitrates present was similar. The introduction of straw to the manure and peat hindered the accumulation of nitrates. Compost number 8 which was made up of 1 part of manure and 12 parts of a mixture consisting of 2 parts of peat and 1 of straw, together with fertilizer salts and lime, contained only 380 parts per million of nitrates.

GREENHOUSE EXPERIMENTS TO DETERMINE THE EFFECT OF PEAT COMPOSTS ON PLANT GROWTH

On October 10 the composts constituting series A were removed, placed on a platform, and thoroughly mixed after which samples were taken for plant studies. The containers used in the plant culture studies were one-

FIGURE 1. Tomato growing in sandy loam + 160 grams composts from series A, Table V. Left to right, composts 6, 5, 4, 3, 2, and 1 sandy loam no treatment.

FIGURE 2. Tomato growing in sandy loam treated with 160 grams composts series A, Table V. Left to right 11, 10, 9, 8, 7, and sandy loam no treatment.

TABLE V

EFFECT OF DIFFERENT COMPOSTS AND FERTILIZERS ON THE GROWTH OF THE TOMATO. SERIES A. DURATION OF GROWTH 36 DAYS

Culture No.	Treatment of sod soil	Av. green wt. g.
1	No treatment	7.2
2	160 g. of wet compost No. 2	57.0
3	160 " " " " " 3	53.4
4	160 " " " " " 4	76.1
5	160 " " " " " 5	95.3
6	160 " " " " " 6	76.1
7	160 " " " " " 7	60.9
8	160 " " " " " 8	92.6
9	160 " " " " " 9	76.9
10	160 " " " " " 10	110.8
11	160 " " " " " 11	57.3
12	80 " " " " " 5	47.1
13	40 " " " " " 10	37.4
14	320 " " " " " 10	136.0
15	320 " " " " " 5	121.5
15A	160 " " " " " 5*	123.0
16	50 " " "driconure"	46.8
17	100 " " "	90.5
19	50 " " dry cow manure	90.5
20	100 " " " " "	103.0
27A	320 " compost No. 1+manure, fertilizers and lime as in culture No. 15	53.1
28	160 g. compost No. 1	13.5

* Four grams of a mixture containing 2 parts of superphosphate and 1 part of KCl.

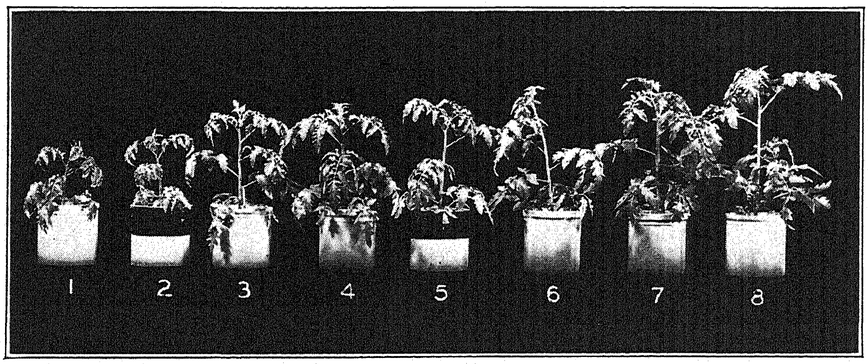

FIGURE 3. Tomato growing in sandy loam. (1) No treatment; (2) Fertilizer salts, lime, peat, and manure as in culture No. 7; (3) Dried stockyard manure, dry matter, as in culture No. 7; (4) 40 grams compost 5, series A, Table V; (5) Commercial sheep manure, dry matter, as in culture No. 7; (6) "Driconure," dry matter, as in culture No. 7; (7) 80 grams compost 5, series A, Table V; (8) 160 grams compost 5, series A, Table V.

gallon glazed jars. Tomato plants (*Lycopersicon esculentum* Mill. var. Bonny Best) were used in the first series. Each culture treatment was replicated six times. The make-up of the various cultures and the average

green weight of six plants are given in Table V. The growth of the tomatoes is illustrated by Figures 1, 2, and 3.

The addition of 160 grams of moist composts 2, 3, 7, and 11 to sod soil (Table V) resulted in large increases in yield of the tomatoes over the untreated mineral soil and the mineral soil to which 160 grams of the untreated peat was applied. Somewhat larger yields were obtained from the application of the same amount of composts 4, 6, and 9. Evidently the most effective composts were 5, 8, and 10. These were superior to equivalent amounts of "driconure" and commercial, dried, cow manure. Composting increased the efficiency of the peat. The same but uncomposted combination as contained in 320 grams of compost 5 resulted in less than one-half the yield of tomatoes as was derived from the same amount of this compost. The addition of 80 grams of compost 5 moreover produced about one-half as much yield as did 160 grams, and the reinforcement of this compost with superphosphate and potassium chloride, as in culture 15 A, reacted favorably.

Rye grass (*Lolium perenne* L.) was also employed to ascertain the effect of composting peat. In this series a medium coarse sand subsoil was employed as the basic medium. One-gallon jars were filled to within two inches of the top with the sand. The composts and other treatments were mixed with sufficient sand to fill the jars to within one-half an inch of the top. The results obtained from the series of cultures are given in Table VI.

TABLE VI

EFFECT OF DIFFERENT COMPOSTS ON THE GROWTH OF RYE GRASS. SERIES A. DURATION OF GROWTH 21 DAYS

Culture No.	Treatment of sand	Av. yield green wt. g.	
		1st cutting	2nd cutting
1	No treatment	2.4	1.1
2	40 g. wet compost No. 2	14.5	4.5
3	40 " " " " 3	12.4	4.5
4	40 " " " " 4	9.6	3.8
5	40 " " " " 5	14.2	—
6	40 " " " " 6	10.4	4.7
7	40 " " " " 7	9.4	4.2
8	40 " " " " 8	10.3	3.0
9	40 " " " " 9	13.3	4.0
10	40 " " " " 10	15.1	7.6
11	40 " " " " 11	11.1	5.0
12	80 " " " " 5	15.3	7.9
13	160 " " " " 5	24.0	18.5
14	12 " "driconure"	5.3	2.4
15	24 " "	7.3	4.1
16	40 " wet peat No. 1	2.5	1.3
17	40 " " " " 1*	3.5	1.4
18	40 " " " " 1**	5.3	2.5
19	40 " " " " 1***	6.3	2.8

* 0.48 g. phosphate, 0.16 g. K_2SO_4, +1.6 g. limestone+manure.
** 0.48 g. phosphate, 0.16 g. K_2SO_4, +0.12 g. $(NH_4)_2SO_4$+manure.
*** 0.48 g. phosphate, 0.16 g. K_2SO_4, +0.24 g. $(NH_4)_2SO_4$+manure.

The first cutting of the rye grass revealed composts 10, 5, and 2 to be slightly superior to the others. Next in order were composts 9 and 3. The remainder were slightly less effective for this plant. The presence of 160 grams of compost 5 produced considerably more grass than did the other cultures. The "driconure" was slightly less effective than the peat to which fertilizer salts, manure, and lime were added, and much less so than the composts.

The residuary effects of the treatments were determined by taking the weights of the second cutting of grass. With the exception of the cultures which contained compost 10, the differences were not great. The yield from the 160 gram application of compost 5 was much larger than those from the cultures which received smaller amounts of this compost. The resid-

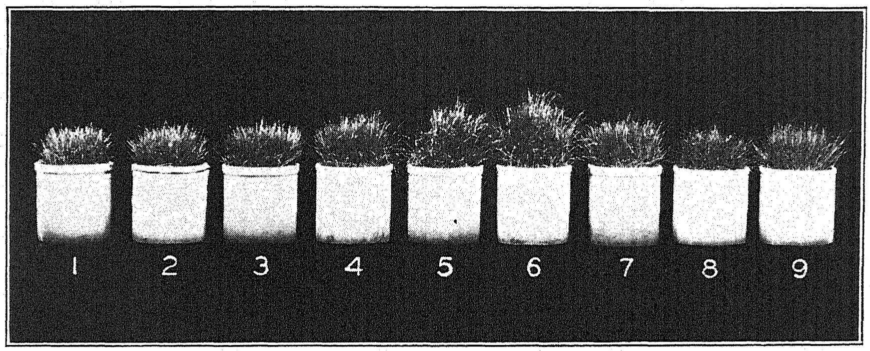

Figure 4. Rye grass growing in sandy loam. (1) No treatment; (2) Fertilizer manure and lime as in 20 grams compost 5, series A, Table I; (3) 10 grams compots 5, series A, Table I; (4) 20 grams compost 5, series A, Table I; (5) 40 grams compost 5, series A, Table I; (6) 80 grams compost 5, series A, Table I; (7) "Driconure" as in culture 4; (8) Commercial sheep manure as in culture 4; (9) Dried stockyard manure as in culture 4.

uary effects of the composts were greater than were those of equal amounts of "driconure." The growth of the rye grass in the cultures which received the same amounts of peat, fertilizers, lime, and manure, as were originally present in compost 5 was inferior to that to which this compost was added. As shown by Figure 4, compost 5, series A was superior to the commercial manures, "driconure," sheep manure, and dried stockyard manure for the production of rye grass.

The comparative value of compost 5 and commercially prepared sheep manure for top dressing purposes was determined by applying them in different amounts to rye grass growing in unfertilized sandy loam soil. As illustrated by Figure 5, the former was slightly superior to the latter.

The results obtained from culture studies of tomatoes and rye grass show that very good mixtures result from composting peat with the proper

amounts of fertilizer salts, manure, and lime. The value of these when used under field conditions may be different from those which have been obtained from the greenhouse investigations. It also remains to be determined how long the mixtures should stand before they should be utilized in order to obtain maximum results.

Samples were taken from the peat and straw composts, series B, on February 17 and their effects on the yield of tomatoes determined as in the

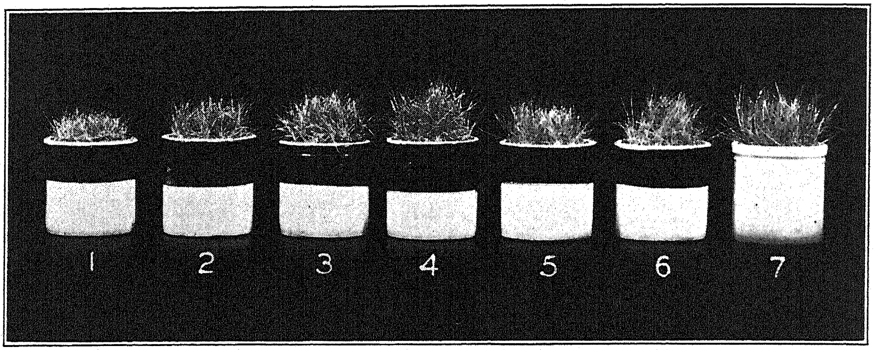

FIGURE 5. Effect of top dressing on the growth of rye grass. (1) Sandy loam soil untreated; (2) Sandy loam soil+20 grams compost 5, series A, Table I; (3) Sandy loam soil+40 grams compost 5, series A, Table I; (4) Sandy loam soil+80 grams compost 5, series A, Table I; (5) Sandy loam soil+dry sheep manure as in culture No. 2; (6) Sandy loam soil+dry sheep manure as in culture No. 3; (7) Sandy loam soil+dry sheep manure as in culture No. 4.

TABLE VII

EFFECT OF DIFFERENT COMPOSTS AND FERTILIZERS ON THE GROWTH OF THE TOMATO. SERIES B. DURATION OF GROWTH 42 DAYS

Culture No.	Treatment of sandy loam	Av. yield green wt. g.
1	No treatment	12.5
2	160 g. wet compost No. 2	67.3
3	320 " " " " 2	97.2
4	160 " " " " 3	64.7
5	160 " " " " 4	66.8
6	160 " " " " 5	71.5
7	160 " " " " 6	87.0
8	160 " " " " 7	46.9
9	160 " " " " 8	70.2
10	160 " " " " 9	86.3
11	160 " " " " 10	6.9
12	80 " " " " 11	51.0
13	160 " " " " 11	69.0
14	160 " as No. 2 not composted	58.9
15	160 " " " 9 " "	59.3
16	"Driconure"*	22.2
17	" **	40.4
18	Commercial sheep manure***	20.7

* Dry matter as in 160 g. No. 2 compost; ** dry matter as in 320 g. No. 2 compost; *** dry matter as in 160 g. No. 2 compost.

previous series. The make-up of the cultures and the average green weight of six tomato plants produced are given in Table VII. The growth of the tomato plants in the cultures to which were added composts 2, 6, and 9, was the greatest. Composts 5, 8, 11, and 3 were somewhat less effective and 7 and 10 were much less valuable. It is obvious that the full quota of lime in the composts was not so efficient as two-fifths of it. Where the lime was omitted, as represented by compost 7, the yields obtained were unsatisfactory. Considerable benefits resulted from composting, as shown by a comparison of the weight of the plants grown in culture 2, which received 160 grams of compost 2, with those grown in 14 to which was added the same but uncomposted materials.

Rye grass also was employed to test the value of these composts. This set of experiments was begun on October 8, or two months after the composts were started. Forty grams of each compost and an equivalent amount of commercial cow manure were added to the sandy loam soil. The grass grew luxuriantly in all cultures to which the composts were added. The yields were superior to those obtained from the use of either cow manure or peat, salts, lime, and manure without composting. The second cutting of the grass yielded approximately 65 per cent as much green weight as the first one.

TABLE VIII

Effect of Different Composts and Fertilizers on the Growth of Rye Grass. Series C. Duration of Growth 24 Days

Culture No.	Treatment of sandy loam	Av. yield green wt. g.
1	40 g. wet compost No. 1	15.6
2	40 " " " " 2	20.4
3	40 " " " " 3	18.4
5	40 " " " " 5	20.0
6	40 " " " " 6	23.0
7	40 " " " " 7	24.8
8	40 " " " " 8	24.0
9	40 " " " " 9	19.6
10	40 " " " " 10	24.0
11	40 " " " " 11	24.4
12	40 " " " " 12	19.6
13	40 " " " " 13	17.6
14	40 " " " " 14	26.0
15	40 " " " " 15	10.7
16	40 " " " " 16	27.0
17	40 " " " " 17	27.0
18	40 " " " " 18	20.0
19	40 " " " " 19	17.8
20	40 " " " " 20	22.3
21	No treatment	2.5
22	Manure, salts, and lime	14.6
23	Commercial sheep manure (dry matter) as in 40 g. compost	10.0
24	40 g. peat No. 5+ salts, lime, and manure	17.0
25	40 " " " 12+ " " " "	15.0
26	40 " " " 9+ " " " "	17.0

The value of the composts in series C was determined in the usual manner with rye grass and tomato plants. The cultures were started February 19, 1932. The results obtained are summarized in Table VIII. The variations in the yields of the grass grown in the differently treated cultures were considerable. The best results were obtained from those which carried the sedimentary, fibrous, sedge peat number 14, the woody reed and sedge peat number 4, the reed and sedge peat number 11, the partly decomposed sphagnum peat number 15, and the woody sedge and reed peat number 8. The sandy and silty organic debris, chiefly sedimentary material with small amounts of woody fragments, or peats 1, 2, and 3, and the reed and hypnum peat number 9, were considerably less effective than the others. The materials taken from the surface of the deposits, with the exception of peat number 1, resulted in larger yields of the grass than did the less weathered materials, as shown by a comparison of results obtained from cultures 20 and 13, 16 and 18, and 17 and 15. The omission of manure, as exemplified by compost 9, resulted in lower yields of the grass. The composts were superior to commercial sheep manure for the production of this plant. It should be noted also that the straw in the various composts was dark in color and easily broken up when handled or when placed in the dispersing machine.

Small differences in the yields were obtained from the cultures which were treated with composts derived from the above described peats (numbers 8, 4, 14, 15) and the reed and sedge peats (numbers 6 and 12). Inferior composts resulted from the use of peats 1, 2, and 3. The same amounts of straw, manure, fertilizer, lime, and peats 12 and 9 were much less effective than were the same combinations after composting. The results derived from cultures 10 and 15 respectively show similar relationships.

The fibrous, sedge peat composts were thoroughly mixed and samples taken for plant culture studies on February 17, 1932. The make-up of the cultures and the yields of rye grass obtained therefrom are given in Table IX. It is to be concluded from a comparison of the yields obtained from cultures 1, 2, 4, 5, and 6, or those in which the ratios of manure to peat were 1 to 1, 1 to 3, 1 to 5, 1 to 10, and 1 to 40 respectively, that the presence of small amounts of manure in the mixture was as effective as large quantities for rye grass. The greatest yield of grass was derived from the application of compost 7, or the one which carried one part of manure and one part of a mixture containing two parts of peat and one part of straw, together with the fertilizer salts and lime. Further dilution with straw as in compost 8 was less advantageous. There appeared to be no correlation between the amount of nitrates present in the composts and the yield of barley, results similar to those reported by Manns and Goheen (5).

These composts were utilized in another series to determine their effect

TABLE IX
EFFECT OF DIFFERENT COMPOSTS AND FERTILIZERS ON THE GROWTH OF RYE GRASS. SERIES D. DURATION OF GROWTH 27 DAYS

Culture No.	Treatment of sandy loam	Av. yield green wt. g.
1	40 g. compost No. 1	29.0
2	40 " " " 2	26.0
3	40 " " " 3	11.0
4	40 " " " 4	28.0
5	40 " " " 5	27.6
6	40 " " " 11	30.4
7	40 " " " 6	33.0
8	40 " " " 7	36.0
9	40 " " " 8	25.0
10	Manure + peat + salts + lime as in compost No. 1	15.0
11	" + " + " + " " " " " 2	22.4
12	" + " " " " " 3	8.0
13	" + " + salts + lime " " " " 4	21.6
14	" + " + " + " " " " " 5	21.6
15	" + " + " + " " " " " 11	27.8
16	No treatment	4.2

on the growth of the tomato plant. Compost number 1 which was made up of one part by weight of manure and one part by weight of moist peat, and compost number 6 which contained one part of manure and one part of a mixture containing two parts of peat and one part of straw were superior to the others. The amounts of fertilizer salts used in making up the composts were too small for the tomato plant, that is, when 160 grams of the composts were applied. This may account for the difference in the response of the rye grass and the tomato plants.

Judging from the results of the greenhouse studies, valuable materials for soil improvement may be obtained by composting certain peats with small amounts of manure and suitable quantities of fertilizer salts and lime. A very suitable amendment for greenhouse soils in these experiments consisted of two parts of a good grade of peat when composted with one part of rye straw, fertilizer salts, lime, and a small amount of manure.

SUMMARY

1. A partly decomposed, sedimentary, fibrous, sedge peat composted with fertilizer salts and different amounts of lime and manure constituted series A. The nitrates were determined 217 days later. The minimum amount of nitrate nitrogen was 933 parts per million of the untreated composted peat. The compost which did not receive manure contained 4124 parts per million. The maximum nitrate attained was 5882 parts per million in the compost made up of 7.5 pounds of ammonium sulphate, 15 pounds of superphosphate, 5 pounds of potassium sulphate, 10 pounds of lime, and three bushels of manure made up to one cubic yard with peat. The compost which contained 7.5 pounds of ammonium sulphate, 15

pounds acid phosphate, 5 pounds of potassium sulphate, 10 pounds of limestone, and 20 pounds of manure per cubic yard of peat, contained 5181 parts per million. These results in general are similar to those reported by Jakuschkin (2).

2. The quantity of nitrates varied widely in the composts making up series B, in which the above peat was composted with straw. The omission of either limestone or manure from the compost resulted in a lowering of nitrate accumulation. The nitrates present in the compost which contained limestone amounted to 8740 parts per million and where it was omitted there were 5870 parts per million. There were 4307 parts per million in the one from which the manure was omitted.

3. Twelve peats were composted with straw, fertilizer salts, limestone, and a small amount of manure in series C. The amount of nitrates present in the composts after 199 days varied widely. The most active composts were those which contained the woody reed and sedge peat 4, the partly decomposed, sedimentary, fibrous, sedge peat 14, and reed and sedge peats in a moderately advanced degree of decomposition or 11, 12, and 5. The composts which contained the remainder of the peats, were comparatively sluggish in the formation of nitrates. The presence of the manure aided in the formation of nitrates.

4. Although the composts in series D ranged widely in the ratio of manure to the partly decomposed, sedimentary, fibrous, sedge peat, the amount of nitrates was not strikingly different, ranging from 7129 to 8865 parts per million. The presence of straw in the manure, salt, lime, and peat mixtures retarded the nitrate accumulation. There were 380 parts per million of nitrates in the compost made up of 1 part of manure to 12 parts of a mixture of 2 parts of peat and 1 of straw, in addition to the salts and lime. Kupryanov and Rozonov report similar results.

5. The partly decomposed, sedimentary, fibrous, sedge peat when composted with fertilizer salts, lime, and a small amount of manure, proved to be a valuable soil improver as measured by the growth of rye grass and tomatoes. It was superior to the same materials without composting. A satisfactory mixture consisted of 7.5 pounds of ammonium sulphate, 15 pounds of superphosphate, 5 pounds of potassium sulphate, 10 pounds of lime, and 20 pounds of horse manure per cubic yard of peat.

6. Straw changed markedly in its physical properties when composted with the above peat and the proper amounts of fertilizer salts and lime. It disintegrated readily upon handling and was more effective for soil improvement than equal amounts of commercial manures. These results cannot be compared directly with those of Collison and Conn (1) owing to differences in conditions under which they were composted; yet they show similar trends.

7. Two parts of each of 12 peats were composted with 1 part of rye

straw, and in addition fertilizer salts, lime, and manure. The sedimentary, fibrous, sedge peat was composted with different amounts of straw, manure, and lime. Great differences were found in the soil improvement values of the composts containing different peats. The presence of a small amount of manure in the composts was as advantageous for the rye grass as was large quantities. It is apparent from these results that if two or more parts of wet peat are mixed with one part of dry straw and placed in heaps, little if any additional water is necessary for proper composting.

8. The ratio of manure to the sedimentary, fibrous, sedge peat was varied from 1 to 1 to 1 to 40. The results showed that peat composted with a small amount of manure, fertilizer salts, and lime, was as effective in the improvement of sandy loam soil for rye grass as it was when composted with manure in much narrower ratios. Narrower ratios of manure to peat were superior for the production of tomato plants.

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COTTON FIBERS. II. STRUCTURAL FEATURES OF THE WALL SUGGESTED BY X-RAY DIFFRACTION ANALYSES AND OBSERVATIONS IN ORDINARY AND PLANE-POLARIZED LIGHT

WANDA K. FARR[1] AND GEORGE L. CLARK[2]

A century of study of the nature and arrangement of cell wall materials has furnished the basis for continuous discussion and controversy. Many issues with respect to walls of comparatively simple structure remain undecided. The lack of agreement concerning the fundamental principles underlying wall formation in instances of slight differentiation serves to accentuate the difficulty of interpreting the more complicated structure of the cotton fiber wall.

A fairly comprehensive index to the literature covering the entire period may be obtained by consulting the early contributions to the anatomy and physiology of the plant cell by von Mohl (27), the discussion of cell wall structure and growth by Strasburger (39), the treatises upon plant cell membranes by Gaucher (19), the monograph upon cell membranes by van Wisselingh (42), the articles upon the structure of the cotton fiber by Denham (14, 15), the survey of more recent work upon plant cell walls by Anderson (4), and the interpretations, based upon X-ray diffraction patterns, of the exceedingly minute structure of the cell wall and the mechanism of its formation by Sponsler (35, 36, 37).

The descriptions and theories presented are related to the living or nonliving nature of the cell wall material; its relation to the living protoplast; its chemical composition; the presence or absence and methods of formation of cell wall lamellae; the spiral, annular, or reticulate wall striations; structural units within the limits of microscopic observation, fibrillae, microsomes, dermatosomes and their arrangement; structural units of submicroscopic dimensions, micellae, cellulose unit cells, cellobiose residues, glucose residues and their arrangement; as well as the possible relationships between the gross physical properties of the wall and its various types of structural differentiation. Many of the most recent theoretical discussions are based upon observations in polarized light and ultra-violet light and X-ray diffraction analyses. The complex physical and chemical nature of cell wall substances, and the degree of variation found in cell wall materials of the tissues of a single organism are among the many important difficulties in the way of establishing general conclusions. In many instances interpretations are based upon comparisons of results with those

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from other substances whose properties are believed to be more clearly understood.

PROBLEM

Previous X-ray analyses (13) of three kinds of mature cotton fibers, having measurably different physical properties, suggested the possibility that consistent variations in the X-ray diffraction patterns of cotton fibers of various types might be obtained. The first set of samples had included a cotton of high "quality," a second whose fibers had been affected by adverse developmental conditions, and a third which represented a distinctly inferior variety. The patterns were made from small bundles of untreated cotton in which the fibers had been arranged parallel to one another. During the exposures, the long axes of the fibers were perpendicular to the X-ray beam. In all three cases the diffraction lines had positions upon the patterns corresponding to those of true cellulose. A difference was observed, however, between the lengths of the chords of arcs making up the diffraction rings. Values of 2.8 cm., 3.25 cm., and 3.8 cm. were obtained from the high, medium, and low qualities respectively, indicating variations in regularity of orientation of the structural units in the wall (10, p. 153).

At the same time microscopic studies of the wall structure of many types of fibers were in progress. Inconsistencies in the regularity of secondary wall configuration were observed in fibers of different varieties, in fibers of the same variety, in fibers from a single seed, and even in different parts of the same fiber. The possibility of a relationship between degree of regularity in gross orientation of the wall materials and the variation in the lengths of arcs produced upon the diffraction patterns suggested itself. In other words, the question arose as to whether or not a wall structure of extreme regularity in a cotton fiber would produce a diffraction pattern with shorter arcs than would be indicated in the diffraction pattern of a wall or portion of a wall less regular in structure. If this were found to be true, it would follow that a measurable relationship may exist between the arrangement of the very small structural units evidenced by means of X-rays, and the variations in wall structure which are within the limits of microscopic observation.

METHODS

A natural classification of mature fibers must necessarily have its foundation in a knowledge of the complete range of variation of the major structural and functional features of the wall materials. Experiments to this end may make use of many different types of technique applied to carefully selected material. It is obvious that any general relationships which may be established between degrees of variation in wall structure and characteristics of diffraction lines must be based upon a knowledge of specific correlations between the two.

One method of approach is through X-ray diffraction patterns of fibers in which the degree of variation in wall structure, as shown by the microscope, has been accurately determined. Since a diffraction pattern is usually made from a bundle of a hundred or more fibers, and since the variations in wall structure within a single fiber are numerous enough to make a sufficiently detailed miscroscopic survey of the irradiated portion of such a bundle very difficult, specific correlations by this means were not attempted.

The production of X-ray diffraction patterns of a single fiber whose areas of more or less uniform wall configuration have been mapped and photographed separately will furnish an excellent basis for specific correlations. The single fiber method requires, however, the use of a very small beam of X-rays which, in turn, makes necessary the construction of a special type of camera. The first model of such a camera has been constructed and is being tested in the X-ray laboratory. Even more accurate information may be expected from similar treatment of very thin pieces of the fiber wall. Dissection of the fiber wall to this end has not yet produced satisfactory results. In the meantime observations which seem to warrant general correlations between the factors concerned are worthy of consideration.

The present report is based upon the study of fibers from a single pure bred strain of *Gossypium hirsutum* L., a representative Upland cotton, grown under controlled nutritional conditions. Comparisons are made with fibers of "Jungle cotton," of unknown origin,[3] whose wall structure has been found to have a high degree of regularity in contrast to the irregular wall structure of cotton fibers.

Microscopic observations and illustrations are limited to longitudinal portions of entire fibers. This will eliminate from detailed consideration the cross-sectional fiber variations. The data are concerned with portions of cotton fibers of the given variety as well as portions of fibers of Jungle cotton in both ordinary and plane-polarized light.

Cotton fibers which were examined microscopically and photographed in ordinary light were slightly swollen in cupraammonium in order to make more clearly visible the details of wall structure. Some of the fibers which were studied and photographed in plane-polarized light were swollen to a similar extent in a saturated solution of ammonium thiocyanate in concentrated ammonia.

Cotton fibers from the same seed were prepared for X-ray diffraction analysis in the following ways: (a) cut into a fine powder with straight, sharp blades and pressed into a soft pellet; (b) cut into a fine powder with

[3] This material is probably composed of bast fibers and was obtained through the courtesy of Mr. M. D. C. Crawford, formerly research associate of the American Museum of Natural History.

blades, ground into a very fine powder in a mortar, and pounded into a thin flake with a pestle; (c) untreated fibers for control specimens.

The untreated fibers of both Upland cotton and Jungle cotton were carefully paralleled and mounted across a rectangular opening in a small cardboard frame. The "soft pellet" and "flake" specimens were supported in a similar frame with narrow bands of adhesive tape. During the period of exposure the cardboard frame was mounted over the pinhole of the X-ray diffraction apparatus, a portion of which is shown in Figure 1, in such a way that the small beam of X-rays passed as nearly as possible through the center of the specimen. The entire series of exposures was made upon the same pinhole. All exposures were made for equal lengths of time. Pat-

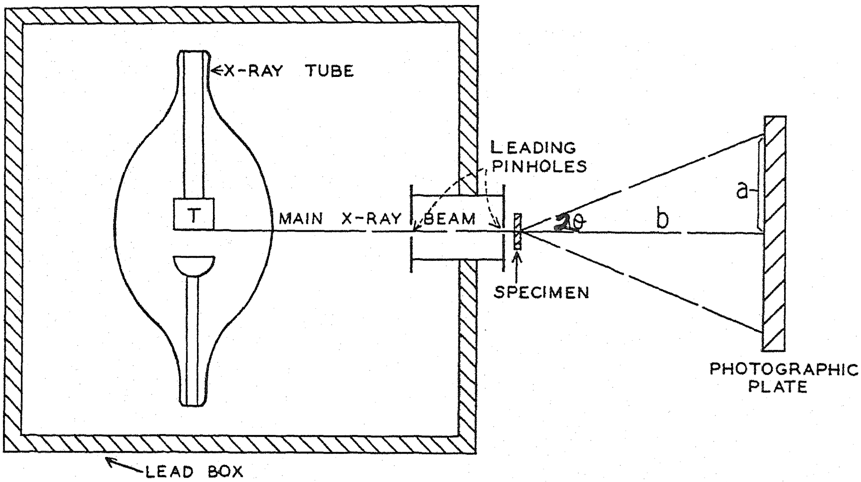

FIGURE 1. Diagrammatic sketch showing the relative positions of the X-ray tube, beam, specimen, and photographic plate in an X-ray diffraction apparatus.

terns of untreated fibers were made with the beam perpendicular to the long axes of the paralleled fibers. Wave lengths of approximately 1.54 Å from a copper target T were employed. The X-rays of the main beam in passing through the specimen were diffracted by the small structural units of the fiber walls in such a way as to produce a pattern upon the photographic plate.

Values for the two factors b and a, Figure 1, representing the distance from the specimen to the plate, and the distance from the center to a ring or spot upon the diffraction pattern respectively, must be determined by very careful measurement. Through these values the angle θ may be found by means of the formula

$$2\theta = \tan^{-1} a/b$$

The value for d, the distance between two successive layers of unit structures, may then be determined by the formula

$$n\lambda = 2d \sin \theta$$

in which λ represents the wave length of the X-rays and n any integer, depending upon the order of the reflection (10, p. 27–55).

TERMINOLOGY

The presence of areas of unequal density having definite geometrical interrelationships may be observed in many types of cell walls. By the middle of the nineteenth century the more conspicuous types of these differentiations as well as the wall lamellae had acquired a prominent place in the literature (26, 34). Typical wall configurations had been designated (27) as spiral, annular, reticulate, and pitted, and abundant evidence of the existence of one or more of these had been found in the tissues of plants of such widely separated families as the Confervae, Equisetaceae, Sphagnaceae, Cycadaceae, Pinaceae, and Orchidaceae. The distribution of any one type of configuration is equally noteworthy. "Spiral" markings may be observed in the elaters of liverworts, in the sporangia of *Equisetum*, in the cells of the leaf and cells of the cortical layers of *Sphagnum*, in the epidermal hairs of the cactus, in particular layers of the seed coat of *Salvia*, in the bast fibers of *Apocynum*, etc.

The choice of a name to be applied to this more or less spiral type of wall configuration seems to be based upon the opinions of the different authors with respect to the true nature of the spirals. If they are considered to be distinct, separable, organic units, they are most frequently referred to as fasciculi fibrarum (1), or fibrillae (4, 28, 32). If observations are not concerned with the separation of the configurations, they are more often mentioned in terms of "streakings," "striations," "stream marks," "strains," etc. (15, 20). In view of the lack of understanding of their method of formation and the relationships existing between them, other authors have made free use of several types of descriptive terms, e. g., Strasburger (39).

When the walls examined may be broken down by either mechanical or chemical treatment into fiber-like components (4, 32), which possess certain other properties characteristic of the fiber as a whole, the use of the diminutive terms fibril or fibrilla would seem to be justified. As long as such separations have not been accomplished in all cellulose walls, however, the fibril is not yet conceded to be a structural unit common to all parts of all wall material. The use of terms which seem to be most accurately descriptive of the material in hand is therefore permissible and will enter into the following discussion.

As a result of extensive research upon starch grains and cell wall material, Nägeli (29, 30) concluded not only that the walls are made up of fibrils of microscopic dimensions, but also that these, in turn, are made up of ultra-microscopic crystalline units which he named micellae. Stras-

burger's interpretations (39, 40) also seemed to necessitate the existence of unit groups of molecules in the wall, to which he gave the name microsomes. These microsomes, when arranged in a linear fashion, produced "striated" walls. Wiesner (41) referred to regularly arranged particles in the wall as dermatosomes. Sponsler (37, p. 335) has suggested that in the process of wall formation "An opportunity was provided for a condensation reaction which would produce long straight chains of residues or cellulose molecules."

In a recent contribution Astbury and Marwick (5) likewise refer to sets of cellulose chains in the wall material of *Valonia ventricosa*. The greater number of contributions to the structure of cellulose walls and that of related materials, however, have referred to these aggregates of cellulose unit cells as micellae (16, 17, 25). It is noteworthy that all agree in the belief of the existence of molecular aggregates of greater magnitude than the cellulose unit cells, made up of groups of unit cells, and as such, having important relationships with the physical properties of the wall material concerned. Researches by Clark and Corrigan (12) with X-rays of greater wave length (about 10 Å from a magnesium anticathode) are concerned with direct measurement of these groups of unit cells. Definite spacings of 85 Å and 265 Å so obtained contribute substantial evidence to the concept of micellae.

The present data corroborate the findings of previous authors with respect to the crystalline nature of the cellulose wall material as well as the aggregation of the cellulose unit cells into larger groups. No additional information has been obtained, however, to influence a choice with respect to the terms micellae or cellulose chains in connection with these aggregations. Future researches with X-rays of suitable wave length to make possible direct measurement of these groups of unit cells may serve to establish the use of one term or the other, or suggest a new term more descriptive of the existing conditions than either. In so far as these groupings enter into the present discussions they may be termed unit-cell aggregates.

RESULTS

MICROSCOPIC OBSERVATIONS IN ORDINARY LIGHT

In connection with the assumption that variations in orentiation of the minute structural units of the fiber wall are responsible for the nature of the diffraction rings in the X-ray pattern, and also that definite axes of structural units fall into line with the main axes of the wall configurations, three factors are of primary importance from the standpoint of microscopic observation: (1) characteristic variations in the arrangement of wall configurations; (2) abnormal wall formation; (3) fiber convolutions.

The microscopic examination of fibers in ordinary light revealed a considerable degree of variation in secondary wall structure. The more or less

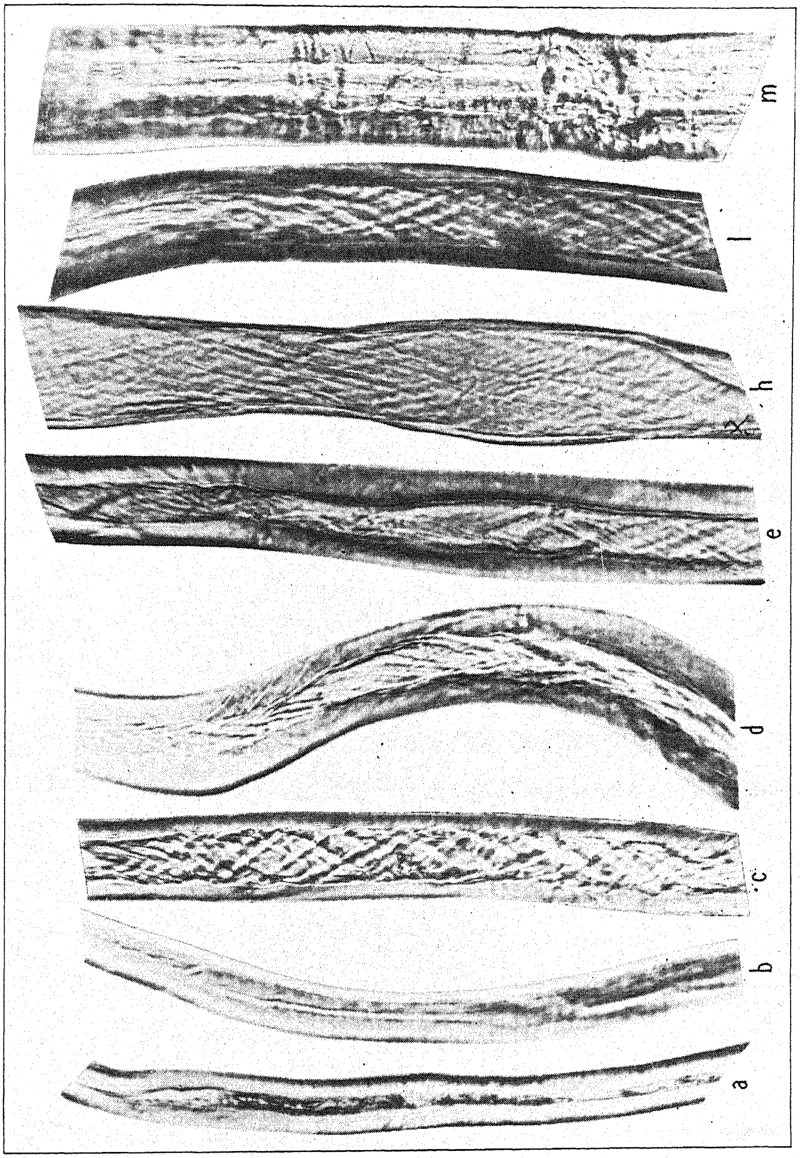

FIGURE 2. Photomicrographs of portions of fibers in ordinary light: *a* to *h* from a single Upland cotton fiber, *l* also from an Upland cotton fiber, *m* from a Jungle cotton fiber. ×1200.

perfect pattern of "double-spirals" to be found in some parts of almost every fiber studied was not observed to continue throughout the length of the fiber (Fig. 2 *e*). Swelling treatment which made more clearly visible

the spiral structure, when present, also served to accentuate the non-spiral nature of striations in other parts of the fiber. In the instances of definite double spiral configuration, the spacing of the ridges was not found to be perfectly regular throughout the region. This irregularity produced such variations in slopes of the spirals and in the angles which the spirals made with one another as are shown in Figure 2 *c*, *d*, *e*, *h*, and *l*.

Although the plane of focus at which spirals are shown in these illustrations lies in some instances in layers of the wall below the surface, it is not believed that in any instance the double spiral effect was produced by bringing into simultaneous focus the striations upon either side of the central canal of the fiber, as shown by Denham (15). This point may be more adequately tested in portions such as are shown in Figure 2 *d* and *e*, both of which are focused a shorter distance below the wall surface than were the portions *c* and *l* in the same figure and by consideration of the degree of magnification itself.

Since the observations were made upon undissected and unsectioned pieces of fibers, the question of whether or not the crossed spirals occur in the same layer of the wall, or owe their appearance to alternation in direction of the spirals in successive layers of the wall material, was not determined. The existence of wall lamellae has been reported in many different types of plant cells and has entered into many theoretical considerations of wall formation. Herzog (23) by means of a swelling reagent observed the wall-layers in the cotton fiber. Their presence in this same material and considerations as to their formation have been the basis for extensive experimentation by Balls (6, 7), Balls and Hancock (8), and Denham (14, 15). Evidence as to their existence in other walls with spiral configurations has come through the microdissection of flax fibers by Anderson (4) and of wood fibers by Ritter (32). Anderson found that each layer of spirally wound fibrils formed a lamellae of the fiber wall and that the direction of the spirals in alternate lamellae is reversed, forming both right and left handed spirals. In longitudinal sections of cotton fibers, Balls (7) found evidence in the secondary wall of spiral reversals at irregular intervals within the same wall lamella. These reversals were of such regularity that the structure on one side of the reversal point seemed to be the exact mirror image of that on the other side. From previous statements and from a consideration of the illustrations in Figure 2, it is obvious that such regularity in the arrangement of wall materials as was observed microscopically by Balls was not found in the present investigation. The distances through which the fibrils maintained a direction parallel to the fiber axis were also found to be greater than those shown by Balls to occur "momentarily" at reversal points of the spirals.

In Figure 2 *a*, *b*, *c*, *d*, *e*, and *h* are portions of a single fiber taken at intervals throughout its length. The portion *h* is representative of the type

of structure found near the proximal end of the fiber. The total thickness of the wall in the basal region of this particular fiber was less than that of the other portions and the appearance in this respect is not due to depth of focus. While the suggestion of spiral striations may be found throughout the length of the entire portion *h*, the lack of continuity and regularity in orientation may also be seen, particularly in those following in the "left handed" direction. The portion *d* is taken at some distance away from the base of the fiber, the portions *e*, *c*, *b*, and *a* following at intervals, in the order given, as the distal end is approached. Figure 2 *e* shows the presence of a very regular spiral area in about one-third of its length, the remaining two-thirds having considerable variation in wall configuration. Figure 2 *c* and *d* have spiral structures throughout their length but vary, in each instance, in their inter-spiral relationships. The portions near the tip of the fiber from which Figure 2 *a* and *b* were taken, presented a very even type of wall formation at all depths of focus with complete absence of the spiral arrangement. Figure 2 *l* is a portion of another fiber and illustrates one way in which a group of spiral fibrils may swing abruptly into a direction approximately parallel to the fiber axis.

The series of illustrations in Figure 2 *a* and *l*, were not selected to emphasize particularly the degree of variation in wall structure. An examination of mounts of a representative number of fibers of this same variety will reveal many cases more extreme than these. In so far as the authors have been able to determine, the types of variation shown will fall well within the range of normality.

The failure to find in the present material the degree of regularity in wall configurations shown by Balls is not to be taken, however, as contradictory evidence. His observations were made upon longitudinal sections of the wall while those here reported were made upon unsectioned and undissected portions of fibers. The most important consideration in this connection may be, however, the different varieties of cotton used in the two instances. That greater regularity would be found in the Egyptian cotton used by Balls than in the American Upland variety used in the present study is not at all unlikely.

Observations of other authors corroborate the present findings with respect to irregular wall structure in the cotton fiber. Denham (15, p. 64) observed that "Such striations do not follow constant lines. Any one set may alter its direction independently of the others and reversals are frequent." Gaidukov (18) mentioned the irregularity of structural units observed by means of the ultra-microscope. Herzog (22) was impressed with this irregularity, and in parts of many fibers, the phenomenon of an abrupt reversal in direction of the spirals, with the intervening zone through which they paralleled the fiber axis, was seen. Ambronn (2) has likewise called attention to the indefinite arrangement of wall materials in the cotton fiber.

In addition to these typical irregularities in internal structure, abnormalities affecting markedly the arrangement of wall configurations with respect to the main axis of the fiber as well as the interrelationships of striations, are numerous in the variety studied. Specific descriptions and illustrations are reserved because of a forthcoming treatment[4] of abnormal wall formations in this same variety. For immediate information concerning the general types of abnormalities to be found in cotton fibers, Denham (15, Pls. VII and VIII) may be consulted.

Convolutions, characteristic of cotton fibers, and distinguishing them from other textile fibers, have been so extensively discussed and illustrated, e. g., by Balls (7), Balls and Hancock (8), and Denham (14, 15), that the considerations with respect to the present thesis are adequately covered. It is obvious that the presence of such convolutions would produce variations in orientation between different parts of the same fiber en masse, thus increasing to some extent in every fiber and to a great extent in a bundle of fibers arranged for diffraction analysis, the degree of irregularity in orientation of structural units.

Microscopic observations in ordinary light show internal variations in arrangement of wall materials, as well as growth abnormalities, and the usual fiber convolutions. A consideration of the precision which enters into the relationship between the position of the structural units of the fiber wall and the X-rays which are diffracted by them (10, p. 1–26) will serve to bring out the importance of even minor irregularities in wall formation. If, as we have assumed, the main axes of the unit-cell aggregates fall into line with the long axes of the wall configurations, and if, throughout the length of a single fiber, there is found a high degree of variation in orientation of these configurations, upon the basis of their visible structural properties, one would expect to produce a diffraction pattern indicative of irregular orientation. Such a diffraction pattern should contrast sharply with one which had had its origin in a bundle of fibers whose walls were indicative of more regular orientation of structural units, in which abnormalities were few, and in which there were no fiber convolutions. Figure 2 *m* represents a section of a fiber of Jungle cotton whose wall structure may be observed to be much more regular than that of cotton fibers. Abnormalities are exceedingly rare and no fiber convolutions are to be found. The irregularities shown in the lower part of this portion are as extreme as any which were observed in any of the fibers in the sample. This wall structure, as well as the strikingly different type of X-ray diffraction pattern produced, furnishes excellent material for contrast with cotton fibers.

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X-RAY DIFFRACTION ANALYSES

The general principles involved in the diffraction of X-rays are essentially the same as those concerned in the diffraction of light. A beam of monochromatic light falling upon a finely ruled surface is reflected in such a way as to form alternate bands of light and darkness. From the spacings of these bands the distances between the rulings may be calculated. These distances, in turn, have a definite linear relationship to the wave length of the light employed. Likewise, in the diffraction of X-rays evenness of particle size in the diffracting material must be associated with X-ray wave spacings of approximately the same linear dimensions.

The phenomenon as produced by light, however, is due to a surface action, while X-rays penetrate the diffracting substance to an extent depending upon its composition and the wave lengths of the rays. The diffraction of X-rays is concerned, therefore, with the space lattice arrangement of the sub-microscopic structural units of the diffracting material and the analysis of the diffraction patterns so produced must take into consideration their three-dimensional nature.

The cellulose which makes up about 90 per cent of the material of the cotton-fiber wall possesses evenness of minute particle size, one of the necessary requisites for the diffraction of X-rays. The compactness of the cellulose unit cells in the walls is similar to that of many other organic substances. Bragg (10, p. 140) describes this as follows: "The density of the crystal is low; there is no 'close-packing'. Rather, the structure is to be likened to a lace-work in space: the bands which join molecule to molecule are localized at definite points of the molecular configuration. If a metal like aluminum can be compared to a pile of shot, an organic substance resembles rather an open girder structure."

For diffraction purposes the wide inter-planar spacings in these organic substances require X-rays of greater wave length than those usually employed in crystal analysis. Copper anticathodes producing wave lengths of 1.54 Å are used instead of molybdenum anticathodes which produce waves less than half as long.

Mature cotton fibers paralleled and mounted in the usual fashion in the path of a beam of X-rays of suitable wave length give interference patterns characteristic of pure cellulose, although chemical analysis reveals the presence of other substances in slight but appreciable quantities. If the distance from the pinhole to the photographic plate are held constant, the values for a, Figure 1, do not change in the patterns from different cotton fiber samples. Correspondingly the calculated values for d are the same, indicating the unvarying nature of the dimensions of the cellulose unit cells concerned. The constancy of the inter-planar spacings in the analysis of the cellulose in many different physical states has served to establish the dimensions of the cellulose unit cell as well as to indicate its resistance

to many types of treatment. The three dimensions from complete analysis, in the present state of correction, according to Sponsler (38), are 10.3 Å, 6.1 Å, and 5.33 Å.

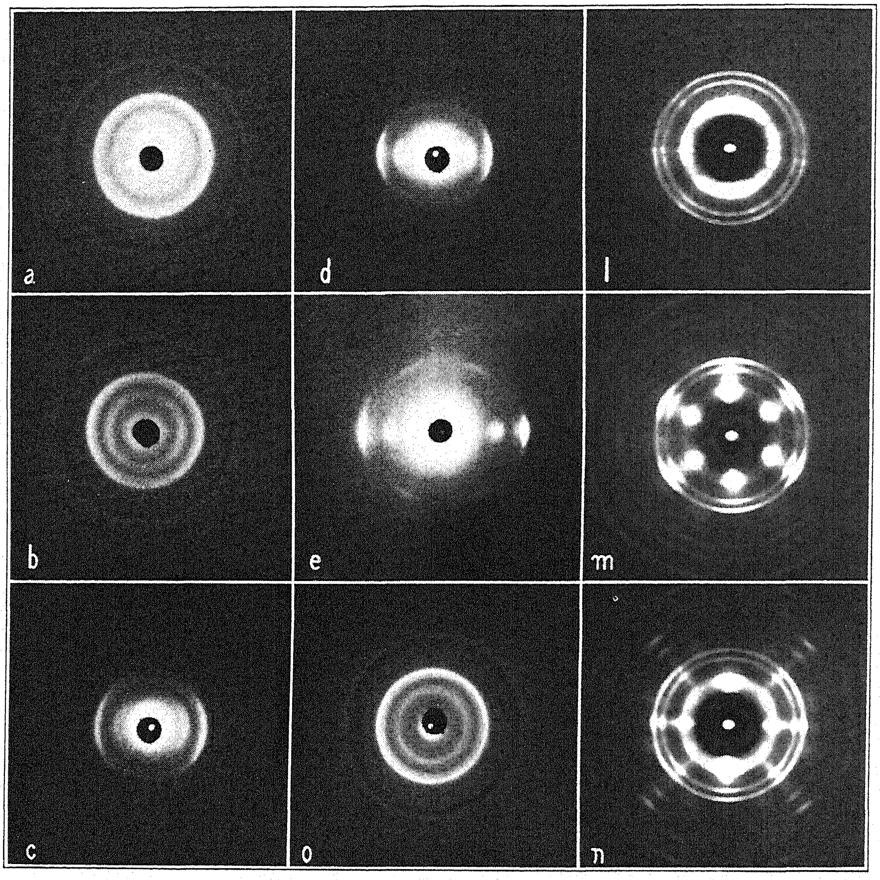

FIGURE 3. X-ray diffraction patterns showing typical "ring," "crescent," and "spot" diagrams in both cellulose and steel: *a*, *b*, *c*, and *d* Upland cotton fibers from the same seed which were powdered, powdered and pounded, untreated, mercerized, and stretched respectively; *e*, untreated Jungle cotton; *l*, *m*, and *n* the powdered, rolled, and stretched states of steel respectively; *o*, the fibril debris resulting from a partial solution of Upland cotton fibers in ammonium thiocyanate solution. (Assistance was received from Miss Julia Southard in preparation of the X-ray diffraction patterns of fibers. The patterns of steel were furnished by Dr. Wayne A. Sisson.)

The spacings of the diffraction lines upon the pattern form the basis for calculation of these unit-cell dimensions. The nature of the lines themselves, however, must be given equally careful consideration. In some in-

stances they are in the form of continuous rings; in others the diffraction rings from the same material after treatment such as stretching are made up of spots of different intensities. Bragg summarizes from large numbers of analyses of crystalline substances made in his own and other laboratories as follows: "A perfect ring photograph implies that the crystalline particles are absolutely irregular in their distribution with respect to the incident pencil of X-rays. If any sort of regularity is imposed upon them, the rings can not be complete." (10, p. 133). "The photograph shows that the orientation of crystals was not complete because each circle can be traced in full, and the spots are somewhat extended along the circle to which they belong. We can imagine every grade of concentration of the spots corresponding with varying degrees of approach to parallelism." (10, p. 154).

In such intensity variations between different parts of the diffraction rings we find a similarity between the patterns of untreated cotton fibers (Fig. 3 *c*), and those obtained from many types of crystalline materials whose structural units have a moderate degree of regular orientation.

In view of these established conceptions and in consideration of earlier results with X-ray diffraction analyses (13) it seemed probable that a series of diffraction patterns of cotton fibers could be made upon the basis of the degree of concentration of spots in the diffraction rings. The position of each pattern in such a series would indicate the relative degree of regularity in orientation of structural units in the sample from which it was made.

The structural regularity found earlier in Jungle cotton by microscopic means (Fig. 2 *m*) was again evidenced in the X-ray diffraction pattern of this same material (Fig. 3 *e*). The Jungle cotton pattern is probably indicative of a greater degree of regularity than may be found in any sample of cotton fibers. For the present, therefore, such a cellulose pattern may be used as an end-point in the series suggested. Figure 3 *c*, from a sample of untreated Upland cotton fibers, falls some distance below the pattern for Jungle cotton. A comprehensive survey will probably locate several stages of untreated fibers in the series between *c* and *e*. Samples from which these intervening stages may be obtained would possess a progressively increasing regularity in structure, approaching Figure 3 *e* as a limit.

The use of different types of cotton to represent positions in the series of lower degrees of orientation than that of Figure 3 *c*, has the present disadvantage of arbitrary selection which obviates specific correlation. The need for these specific correlations as well as methods of obtaining them have been discussed earlier. In the meantime it is of interest, in order to complete the descending scale of the series, to adopt a method of established usage in many other cyrstalline materials (10, Chapter VI, Pls. VII and VIII).

Figure 3 *l*, *m*, and *n* represent the powdered, rolled, and stretched wire states of steel respectively. It is also clear that they represent a complete

series in regularity of orientation of crystalline structural units in that they pass from the continuous "ring" diagram of powdered material, through the "crescent" diagram of rolled material, to the "spot" diagram of the stretched wire.

Theoretically, therefore, the desired stages in the cotton fiber series falling below Figure 3 *c*, should be produced through progressively increasing the degree of irregular orientation by mechanical means. Figure 3 *a* and *b*, represent diffraction patterns of samples so treated. Repeated cutting with sharp blades until the strand of fibers has been powdered produced the state of random orientation indicated in Figure 3 *a*. This is a typical "powder" diagram, comparable in its continuous diffraction rings to Figure 3 *l*. When a neighboring tuft of fibers from the same seed was cut, ground into a very fine powder, and subsequently pounded into a thin flake or "foil," the diffraction pattern shown in Figure 3 *b* was obtained. The appearance of slight differences in intensity in the various parts of the diffraction rings indicates that the pounding of the powdered fiber material had brought about a reorientation of structural units showing more regularity than the powdered sample and less regularity than the untreated paralleled sample from the same seed (Fig. 3 *c*).

A fourth sample of fibers was taken from the same seed, paralleled, slightly mercerized for two hours in 10 per cent NaOH, and stretched. The size of the crescents upon the diffraction pattern (Fig. 3 *d*) indicates that the degree of regularity in orientation had been increased beyond the normal by this treatment, and that the type shown in Figure 3 *e* had been approached.

The diffraction patterns in the entire series *a* to *e* have general characteristics in the nature of their diffraction rings similar to those found in the series *l* to *n*, Figure 3. The cotton fiber material apparently responded to powdering and pounding in a fashion directly comparable to that of steel. These results will confirm the idea of the crystalline nature of the fiber wall materials, and furnish evidence that in the fiber patterns *a* to *e*, we have a definite progressive departure from random orientation.

We may observe again, at this point, the unchanged position of the diffraction rings in the series *a* to *e*. Apparently the cellulose unit cells themselves have remained intact throughout the various types of treatment. It is assumed by adherents of both the "chain" theory and the "micellar" theory that primary valences operate in the end to end linkages of the unit cells or aggregates of unit cells. The severing of large numbers of these bands in the process of powdering the fiber permits greater irregularity in the orientation of the unit-cell aggregates and in turn of the unit cells themselves. That the fibers have been broken into unit-cell aggregates and not into unit cells is evidenced by the sizes of the particles which are visible microscopically.

The diffraction patterns, Figure 3 *a* and *b*, have much in common with the patterns of low and medium quality cotton obtained in the previous experiment (13). If this resemblance is due to similar degrees of regularity in orientation of the structural units, future inquiry with respect to fibers of different "quality" should include, in addition to the single-fiber studies suggested, a consideration of the factors controlling the deposition of cellulose materials during the period of wall formation. Although many explanations of this phenomena have been offered, none of them, in our present state of knowledge, seems to cover satisfactorily the observations in connection with wall formation (28, 30, 37, 39). The inadequacies of the purely mechanistic theories are evidenced in the reference of recent workers (5, 37) to the operation of a "third factor" or "vital" principle.

MICROSCOPIC OBSERVATIONS IN POLARIZED LIGHT

Many crystalline and colloidal substances exhibit different optical properties in different directions. They are referred to as anisotropic substances in contrast to isotropic materials which exhibit identical optical properties in all directions. Vibrations of light, upon entering an optically anisotropic substance, are resolved into two components which vibrate only in planes perpendicular to one another. Since these component vibrations travel at different rates, they have different indices of refraction. When a doubly refractive material is placed between crossed nicol prisms[5] in such a way that the planes of vibration of the specimen are in alignment with those of the crossed prisms, it appears dark and is said to be in a position of *extinction*. In this position it gives no evidence of its doubly refractive character. By rotating the material between the crossed nicols it is observed that there are four extinction positions at 90° intervals and that between these extinction positions the material appears bright on a dark field. In a position of brightness, the two component vibrations which pass through anisotropic materials travel with different velocities, the slower one suffering *retardation*. In an instance of retardation some of the constituent wave lengths of white light are destroyed. The remaining wave lengths produce *polarization colors* characteristic of the material which is being examined. In a single crystal, the amount of retardation and consequently the polarization colors shown may be affected by the thickness of the specimen, by the difference in the relative refractive indices of the two components of the doubly refractive material, or by the orientation of the crystal which determines the direction in which the light travels through it. The polarization colors vary in definite series in direct proportion to the amount of retardation resulting from any one of these

[5] Ambronn and Frey (3) and Chamot and Mason (11) may be consulted for theoretical considerations in connection with polarized light as well as its application to the study of various materials.

EXPLANATION: FIGURE 4

Hand colored photomicrographs of portions of fibers in plane-polarized light: *a* to *r* from Upland cotton, *s* and *t* from Jungle cotton; long axes of portions oriented at 45° with reference to the plane of vibration of the light; *d*, *n*, *o*, and *r* slightly swollen in a solution of ammonium thiocyanate; *a*, *b*, *c*, *l*, *m*, *s*, and *t* photographed with the analyzer; *d*, *n*, *o*, and *r* without the analyzer; *c* and *m* colored with the selenite plate (red of the first order); *a*, *b*, *d*, *l*, *n*, *o*, *r*, *s*, and *t* without the selenite plate; *a*, *b*, *c*, *l*, and *m* ×690; *d* and *n* ×810; *o*, *r*, *s*, and *t* ×1200. (The coloring of the photomicrographs was done by Miss Flora White, an artist of the Boyce Thompson Institute.)

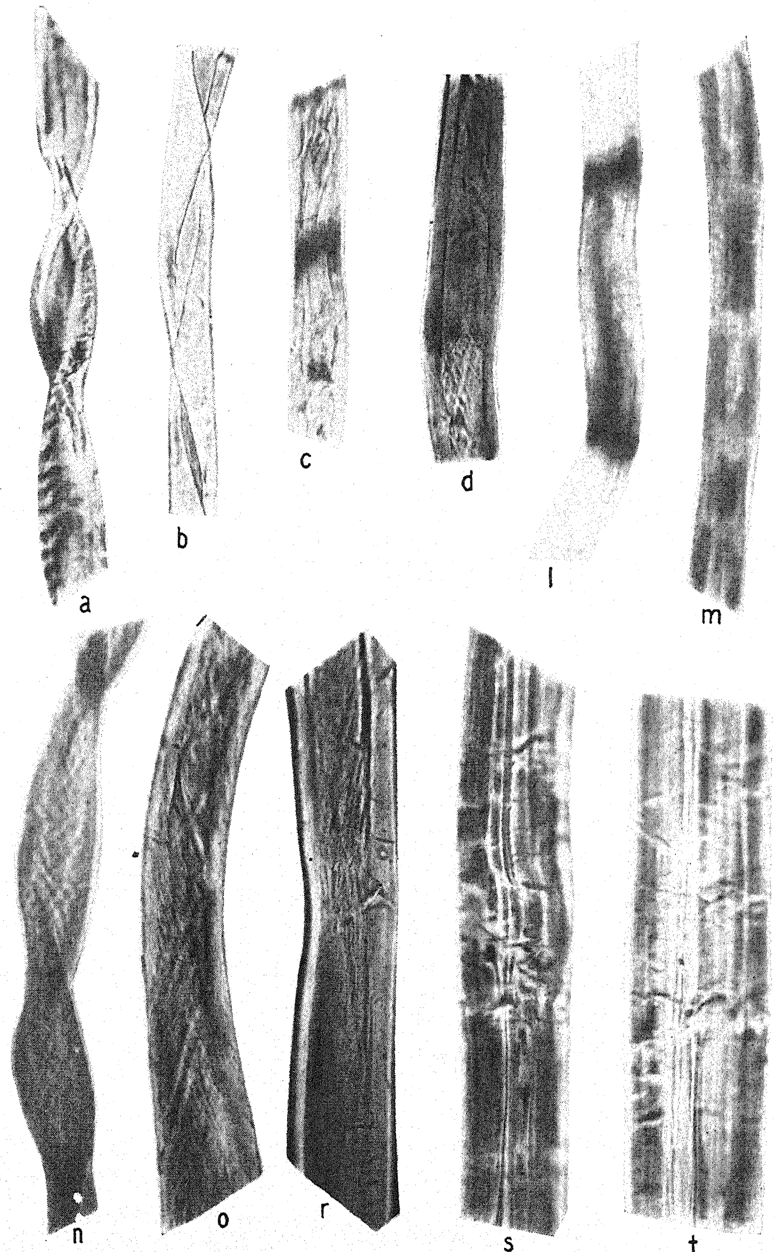

FIGURE 4—PORTIONS OF FIBERS IN PLANE-POLARIZED LIGHT

factors. They may, therefore, be used for measurement of retardation by comparison with standard charts (11, p. 281).

The double refraction of cellulose has been recognized and studied by experimenters in both biological and optical fields of research. The extensive researches of Ambronn (2) and Frey (16, 17) are especially noteworthy. Nägeli (30), Gaidukov (18), Harrison (20), Ritter (32), Anderson (4), Herzog (21), Balls (7), and many others have contributed to the records of observations in different kinds of wall materials. In some instances it has been possible to break down the cell walls by either chemical or mechanical means into smaller fiber-like particles (4, 5, 32). One may then observe that these fibrils themselves are doubly refractive, exhibiting the properties of a single crystal.[6] In the cotton fiber, therefore, one is dealing presumably not with a single crystalline structure, but with an aggregate of anisotropic particles. In this connection Chamot and Mason (11, p. 304) state that "Aggregates of anisotropic particles will not exhibit double refraction unless these particles are arranged in some systematic manner so that their effects are cumulative. Otherwise the anisotropy of each tends to compensate that of the others and an isotropic system results."

Untreated cotton fibers of the variety used in the present investigation in plane-polarized light between crossed nicols, exhibit the polarization phenomena generally characteristic of cellulose fibers and many more which, in our present stage of knowledge, do not seem to fall within the range of accurate interpretation. Varying color effects are obtained, some of the most common of which are illustrated in Figure 4 *a* to *s*. In the preparation of the photomicrographs for this figure it was found that in some instances clearer negatives were obtained when the analyzer was removed during the exposure. Consequently *a*, *b*, *c*, *l*, *m*, and *s* were photographed with the analyzer and *d*, *n*, *o*, and *r* without the analyzer. Each mount was kept in place upon the microscope and the optical system of the photomicrographic apparatus was unchanged while a satisfactory print was being made. The analyzer was then replaced and, when colors of a low order were concerned, the selenite plate of the first order red was inserted. The print was then colored from the image upon the ground glass of the camera and the colors carefully checked against the original.

Figure 4 *a* represents a portion of an untreated Upland cotton fiber with typical fiber convolutions. The color is predominately first order blue except at the reversal points where first order yellow is seen. These mass color effects may be reversed by rotating through 90°. According to cur-

[6] Residues from cotton fibers treated with saturated solutions of ammonium thiocyanate according to the method of Scherer (33) have been found to contain large numbers of cellulose fibrils which show the uniform coloration of single crystals. X-ray diffraction patterns of these residues show continuous rings (Fig. 3 *o*), indicating random orientation. Further microscopic and polarized light studies are now in progress.

rent interpretations, the two predominating colors, blue and yellow, at the turning point of the convolution, are due either to differences in thickness or to differences in orientation of unit-cell aggregates, or to both. It may also be observed that both the blue and yellow areas are flecked with a reddish-orange coloration and in two or more places with shades of violet and green. Difficulty was encountered in an attempt to determine the approximate order in the series of these smaller areas of colors, probably because of proximity of the different colors as well as the lack of wide experience in interference color determination.

Figure 4 *b* illustrates another type of convolution in which a flattened ribbon-like fiber is twisted into a long "curl." The areas of first order blue and yellow are slightly tinged with other colors and the dominant color variations themselves would seem to be due to differences in thickness, the blue areas representing one thickness and the yellow areas two thicknesses of the fiber.

Figure 4 *l* is made up of first order blue and first order yellow-white. On the border lines between the three areas of color are two bands which exhibit no contrast in brightness to the dark field and are interpreted as extinction positions. In this illustration there is an absence of both variations in thickness in fiber convolutions. The interpretation of the color effects is no less difficult than in Figure 4 *m*, where the patches of color alternate at shorter intervals. The predominating colors in the latter are first order blue and second order violet. Although the areas of color are sharply delimited as in Figure 4 *l*, the phenomenon of extinction on the border lines between the areas is not so conspicuous. The streaks of color variation in the central region running parallel to the fiber axis are believed to be due to differences in thickness.

There seems to be little basis for interpretation of the presence of these patches of color in Figure 4 *l* and *m*, until a portion of a fiber is found in which the wall configuration and the coloration may be observed simultaneously. Figure 4 *c* illustrates an apparent relationship between the arrangement of the visible wall striations and the areas of different color. Blue of the first order predominates where the striations are oriented at angles with the fiber axis, and a distinct violet where they are more nearly parallel to the fiber axis. The correlation between structure and coloration is more clearly shown in Figure 4 *d*. The border lines between the areas of different wall structure and of different coloration are sharply coincident. In Figure 4 *n* uniformity in color is seen to accompany the high degree of uniformity in structure. The phenomenon discussed earlier in connection with Figure 4 *a* is again shown at the reversal points of the convolutions.

Figure 4 *o* and *r* present a degree of complexity of color patterns which may be found abundantly in any mount of fibers of the variety concerned. In Figure 4 *r* the areas of color seem to have at least a general relationship

to the arrangement of the striations. This is particularly true of the blue and yellow areas in the lower part of the fiber portion. At the turning point of the striations slightly above the central region first order reddish-orange appears, and continues in such a way as to complicate from the standpoint of interpretation the reversal of colors which is suggested as an accompaniment of reversal in direction of striations. Figure 4 *o* is typical of the many areas in fibers in which a clear relationship between configuration of wall materials and interference colors is not obvious.

An explanation of the interference colors produced in portions of Jungle cotton fibers (Fig. 4 *s* and *t*), may be based upon these general relationships between interference colors and structural variations in the cotton fiber. Both microscopic observations in ordinary light (Fig. 2), and X-ray diffraction patterns (Fig. 3), have indicated a greater degree of regularity in orientation of structural units in Jungle cotton than in Upland cotton. It may be suggested, therefore, that the uniform color in the portion of Jungle cotton fiber shown in Figure 4 *t* is associated with this regularity in arrangement of unit-cell aggregates. Figure 4 *s* shows a portion of the same fiber which had been subjected to lateral pressure. The region of the fiber from which this exposure was obtained was attenuated. Crushing in a direction perpendicular to the fiber axis had produced a folding of the wall material along lines parallel to the fiber axis. As a consequence the relative thickness in different regions as well as the orientation of structural units had been altered in the same direction. The color variations are also seen to follow lines perpendicular to the direction of applied pressure.

Herzog (21) in 1904 observed in flax fibers that the vibration axes of the optically active particles made an angle with the fiber axis falling between 0° and 45°. He suggested the probability that this direction fell into line with the outer oblique stripings of the secondary wall thickenings. In 1909 Herzog (22) reported the same general relationships between wall configurations and polarization phenomena in cotton fibers. Behrens (9) found higher polarization colors due to greater optical thickness when the smaller diameter of the fiber was perpendicular to the line of vision. Herzog (24) has made use of this principle in the determination of variations in thickness of artificial silk fibers by means of polarized light.

Balls' more recent results and interpretations (7) are in keeping with those previously suggested. By means of polarized light he was able to map the fiber throughout its length with respect to extinction positions and areas of coloration. He points out that the examination of the interrelations of extinction positions for right and left handed spirals shows that they are constantly related to the fibril axis and not to the cell axis. Ritter's results (32) are likewise corroborative.

Preston (31) pointed out a relationship between the polarization colors and the chains of cellulose unit cells in the wall layers of *Valonia ventricosa*

and *Valonia utricularis*. The large numbers of color patches coincident with the variations in direction of wall striations were correlated directly with the X-ray diffraction results of Sponsler (38) in the same material. Through a later communication of Astbury and Marwick (5), Preston has reported that these discontinuities previously found by him have no influence upon the main features of the X-ray photograph and are not universally present in pieces of *Valonia* examined. Astbury and Marwick upon the basis of microscopic observations in polarized light as well as X-ray diffraction studies find that the wall material of *Valonia ventricosa* is made up of two main sets of cellulose chains, crossing at an angle which is maintained remarkably constant throughout the wall thickness and over considerable areas. The orientation of the cellulose chains was found to be parallel to the direction of the fine, crossed striae visible in the wall. The inter-fiber angle and the proportion of cellulose chains associated with each orientation apparently determined the extinction position.

These considerations with respect to relative proportions and constancy of angles in the arrangement of wall configurations may prove to be helpful in interpreting the multi-color effects in such portions of fibers as are shown in Figure 4 *o* and *r*. Likewise in Jungle cotton the basic structure may be that of very fine, crossed striae whose constancy in proportions and arrangement result in the uniform color observed in polarized light.

If it were possible to separate the wall layers of the cotton fiber into hollow cylinders by methods similar to those used by Anderson (4) and Ritter (32) for flax and wood cells respectively, the questions involved would be more easily clarified. If these cylindrical pieces were severed along a line parallel to the fiber axis, the wall fragment might be flattened into a single layer. The relationships of interference colors, wall striations, and X-ray diffraction phenomena could then be studied as in the wall layers of *Valonia* without the complications introduced by the many layers of the undissected wall.

The separation of striated portions of the wall into fibril-like structures which, in themselves, possess anisotropic properties, produce X-ray diffraction patterns, and may be measured and manipulated microscopically, suggests another avenue of approach to the problem of relationship of gross structure to orientation of minute structural units in the fiber. By sufficiently delicate manipulation the small fibrillar strands might be arranged in the field in such a way as to produce the various interference colors seen in the intact fibers. Successful results would carry with them the usual relative values of synthetic methods.

Microscopic observations in both ordinary and plane-polarized light as well as X-ray diffraction analyses may be considered to be equally important in determining the degree of variation in both the nature and the arrangement of cotton fiber wall materials. Data obtained from suggested

variations in these forms of technique will contribute much to the accuracy of our information. Results of the present attempt at correlation are promising and it seems possible that a sufficiently detailed and comprehensive application of these methods may aid in the solution of many of the problems concerned with the quality of untreated cotton fibers.

SUMMARY

Since previous results with X-ray diffraction analyses of cottons of different "quality" had suggested a consistent variation from the low to the higher qualities with respect to the regularity in orientation of minute structural units in the wall material, fibers from a single seed of a pure bred strain of *Gossypium hirsutum* L. were subjected to X-ray diffraction analyses and microscopic observations in ordinary and plane-polarized light.

Microscopic observations in ordinary light revealed a considerable degree of variation in the configuration of wall materials.

The problem of establishing a relationship between the degree of variation in configuration of wall material and the degree of regularity in orientation of these minute structural units of the wall is outlined.

Difficulties in technique delaying the arrangement of a series of types of cotton based upon such variations in orientation, through specific correlations, are discussed.

A series, similar in its fundamental principles to the one desired, is developed by manipulating the fibers from a single seed in such a way as to produce a powder, a flake, and a bundle of stretched mercerized fibers, and forms a basis for correlations of a general nature.

A diffraction pattern of "Jungle cotton" fibers is used in the series as an example of the highest degree of regularity in orientation. The stretched mercerized fibers fall immediately below the Jungle cotton, the untreated flake and powder samples coming next in their turn in the series of diffraction patterns based upon regularity in orientation of structural units.

The series of diagrams of fiber cellulose are observed to be similar, in the nature of their diffraction rings, to a series of diagrams made from steel in the powdered, foil, and drawn wire states.

Comparison with the steel diagrams confirms the idea of the crystalline nature of the fiber wall material and demonstrates the relationship which exists between the lengths of the arcs upon the diffraction patterns and the degree of regularity in orientation of cellulose unit cells.

The particle size of the powder and flake samples of wall material, as well as the diffraction pattern of fibrils into which striated portions of the fibers may be separated, indicate that these degrees of regularity are brought about through orientation of unit-cell aggregates.

The use of polarization colors as an index of the degree of regularity of

orientation of structural units is considered. The need for specific correlations in this connection is discussed and methods of obtaining them suggested.

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MOVEMENT OF MOSAIC VIRUS FROM PRIMARY LESIONS IN NICOTIANA TABACUM L.

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INTRODUCTION

The spread of tobacco mosaic virus from the area of inoculation to other parts of the host plant has been studied recently by a number of investigators. McCubbin and Smith (10) and Böning (2) determined the average rate of movement over certain paths. Holmes (5, 6) and Samuel (12, p. 504) showed that the virus spreads slowly for some days after inoculation, but at a rapid rate later as it passes along veins and stems. Caldwell (3, 4) found that the virus of aucuba mosaic of tomato does not normally enter the xylem stream, but that it may be induced to enter and move in the xylem by introducing it into a cut petiole; while in the xylem, the virus is incapable of affecting the plant unless parts of the plant containing the invaded xylem are crushed. It is not yet known which cells are normally involved in the movement of virus in the systemic infection. Some information about the path of movement of virus from the inoculated leaf has been secured by making successive measurements of the virus present in different parts of inoculated plants (5), but the difficulty of making enough measurements of this kind to determine the exact path taken by the virus has limited the usefulness of this method of studying the movement of the virus within infected leaves. In a recent paper (6) it was shown that the location of primary lesions of tobacco mosaic and the approximate path taken by the virus in leaving the inoculated area may be demonstrated by staining infected leaves with iodine.

The purpose of this paper is to describe the results of applying the iodine staining technique to the study of the path taken by virus of tobacco mosaic in early stages of the systemic infection in *Nicotiana tabacum* L. var. Turkish, to show modifications in the path of virus in leaves of experimentally treated plants, and to report some factors influencing the time required for virus to reach and produce symptoms on leaves at the top of the plant.

METHODS

The method used to prepare leaves for staining was that described previously (6). At suitable intervals after inoculation young plants were taken from the greenhouse during the afternoon. They were kept in a dark room at 10° C., usually from 5 p.m. to 9 a.m., and then transferred to a dark compartment at 22° C. The presence of virus caused abnormal starch retention in leaves of plants so treated. After six to eight hours at 22° C. leaves were out from the plants, and immersed in 95 per cent ethyl alcohol. They were allowed to remain in the alcohol overnight, or as much longer

as was needed for the removal of their chlorophyll. The leaves were then stained in an aqueous solution of iodine in potassium iodide (60 g. KI and 20 g. I dissolved in 3 liters of water) for some hours, usually four or more, and finally rinsed in water to remove excess iodine and facilitate examination. Staining with iodine served to detect starch in affected tissues. The object of keeping plants at 10° C. for 16 hours before storage at a higher temperature was to avoid too great loss of starch before morning, since young plants stored more than 12 hours at 22° C. in darkness generally lost almost all the starch from their infected leaves, even from areas affected by virus.

A method suggested by Samuel (12) was found very useful for preserving stained leaves. The specimens were dried between sheets of waxed paper inserted between blotters in a botanical press. They formed an easily accessible history of experiments.

The tobacco mosaic virus used in the experiments reported in this paper produces intense mottling and severe distortion in infected plants of *N. tabacum*. It is believed to be similar to the virus used in the past by many investigators, who have referred to it as typical or field type tobacco mosaic virus. Its reactions on a considerable number of host plant species are described at some length in another paper (7).

The plants of Turkish tobacco used for infection were grown in a greenhouse in which the night temperature was generally not allowed to fall below 70° F., but in which the daytime temperatures and the moisture content of the air were not accurately controlled.

EXPERIMENTAL RESULTS

RELATION OF NUMBER OF PRIMARY LESIONS TO ONSET OF SYSTEMIC INFECTION

Observation of previous experiments had shown that plants simultaneously inoculated with small amounts of virus varied considerably in length of time required to show the onset of systemic infection, as indicated by the appearance of clearing of veins, abnormally light color of tissues adjacent to veins. Frequently, for example, the time of appearance of clearing of veins on different plants inoculated in this way varied between four and nine days after inoculation. Under similar environmental conditions heavily inoculated plants might all show clearing of veins within a single day, the fourth after inoculation for example. This effect was recently mentioned by Samuel (12, p. 500). In the experiments described in this paper it was necessary to take into account this effect of the number of primary lesions on the onset of the systemic infection.

A study was therefore made with the object of attaining some control of the period between inoculation and the appearance of systemic symptoms. In one experiment 400 plants were arranged in four comparable sets

of 100 each. The first set of 100 plants was inoculated by five pin punctures on one leaf about half way between base and apex, the second set by 20 pin punctures similarly located on a comparable leaf, the third set by rubbing a similar area of one leaf, and the fourth set by rubbing similar areas on each of four leaves. Each successive inoculation in this series was designed to be more severe than the preceding. All inoculations were made with fresh undiluted juice of mosaic plants, and it was estimated that the lightest method of inoculation gave in most cases one or two primary lesions for each infected plant, and that the heaviest inoculation gave more than a hundred primary lesions for each plant.

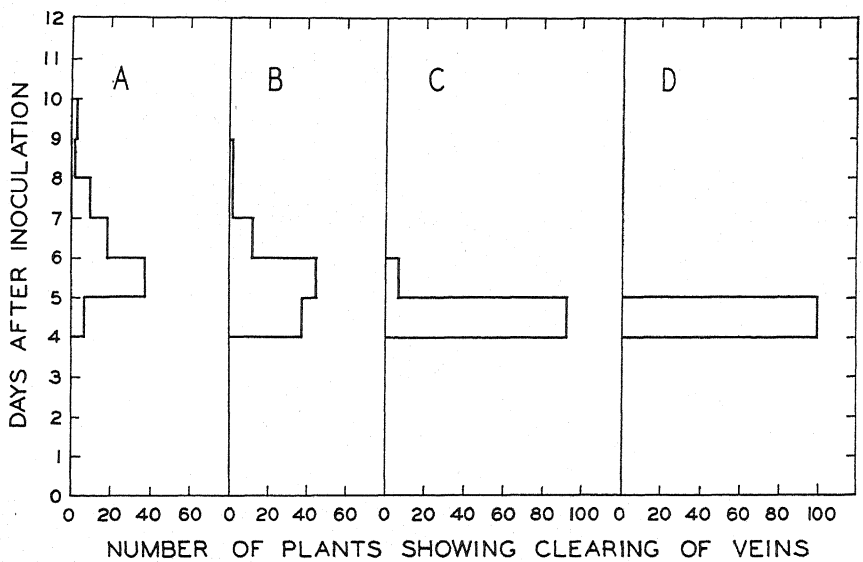

FIGURE 1. Number of plants of *Nicotiana tabacum* var. Turkish showing first systemic symptoms on successive days after inoculation of four sets of 100 plants each: A, Set inoculated by 5 pin punctures on one leaf of each plant; B, 20 pin punctures on one leaf; C, Rubbing on one leaf; D, Rubbing on four leaves.

The details of the resulting incidence of systemic infections are shown in Figure 1. In the first set, which was inoculated lightly, only 78 of the 100 plants became infected. The earliest signs of the individual systemic infections were noted over an extended period, a large proportion of the new cases appearing on the sixth day after inoculation or later. In the second set, inoculated with a larger number of pin punctures on a similar leaf, more plants became infected, and fewer long delayed cases of systemic infection appeared. In the third set, inoculated heavily by rubbing a small area of a single leaf comparable to that receiving pin punctures in the first and second sets, all the inoculated plants became infected, 93 per cent showing

systemic symptoms on the fifth day after inoculation, and 7 per cent the next day. In the fourth set, inoculated by rubbing a small area on each of four leaves of each plant, all the inoculated plants showed symptoms of the systemic infection on a single day, the fifth after inoculation.

The above described experiment furnished data showing the variation in time required for first appearance of systemic symptoms on individual plants given minimum inoculation, and the more nearly simultaneous appearance of systemic infections in plants given heavier inoculation. It is sometimes necessary to have the systemic disease develop nearly simultaneously on a number of plants; heavy inoculation aids in this. It is also necessary to take into consideration the effect of number of primary lesions when studying the time required for the virus to spread in the plant.

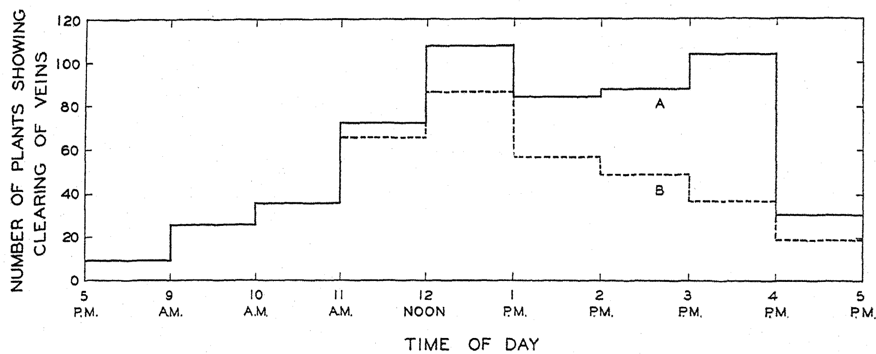

FIGURE 2. Hourly incidence of first appearance of clearing of veins, summarized from data obtained from four sets of plants of *N. tabacum* var. Turkish: A, In all plants (total 554); B, In all plants except those showing symptoms on first day of appearance of clearing of veins in each set (total 378).

FIRST APPEARANCE OF CLEARING OF VEINS

Incidence by hours. In the studies described in this paper, in which the time of appearance of the systemic infection was noted by daily counts of plants showing clearing of veins for the first time, it seemed important to record the number of new cases at that part of the day during which such cases were appearing least rapidly. The time of appearance of clearing of veins was therefore studied in August 1931 and February and March 1932.

Four sets of lightly inoculated plants were observed hourly during daylight hours of successive days, and the time of appearance of clearing of veins on individual plants was recorded. The time (Eastern Standard Time) of appearance of clearing of veins on 554 plants is represented by the unbroken line in Figure 2. Two maxima appear, one at 12 noon to 1 p.m. and the other at 3 p.m. to 4 p.m. The second maximum was due to the fact that on the first day on which clearing of veins was observed in each set no symptoms appeared until a late hour, and then many plants

developed symptoms within a few hours. It was thought that this might possibly be a result of the original hour of inoculation. Since records taken for the night following this day and for later days were distributed more widely through the daylight hours, it seemed desirable to consider also the distribution of instances of onset of systemic symptoms with the records of this first day excluded. This distribution is represented by the dotted line in Figure 2, which shows a single maximum at 12 noon to 1 p.m. Considering only the cases represented by this dotted line, it will be noted that less than 3 per cent of the total of 378 appeared during the 16 hours following 5 p.m., as opposed to more than 20 per cent appearing in the single hour between noon and 1 p.m. This suggests that records made between 5 p.m. and 9 a.m. would be least affected by minor environmental disturbances. When only one observation was made on each day, for the experiments recorded in this paper, it was made between 5 and 6 p.m.

Effect of light. The incidence of clearing of veins during the hours of the day and night, as recorded above and shown in Figure 2, suggested the possibility that light was one of the factors responsible for the phenomenon. It was found that clearing of veins did not occur in plants covered to exclude light, although virus reached the leaves which would ordinarily show this symptom.

The part played by light in causing clearing of veins was tested by shading plants for four days beginning on the third day after inoculation. In full sunlight during June the cleared veins were relatively broad, diffuse in their outlines, and leaves were puckered. In slight shade the cleared areas along veins were narrower and more sharply defined, and the contrast was greater because the green color of intervenal areas was intensified. In deeper shade the cleared areas along veins were inconspicuous, narrow, and not sharply in contrast to the green intervenal tissues.

The evidence given in this and the preceding section indicates that the first appearance of clearing of veins in inoculated plants almost always occurs between 9 a.m. and 5 p.m., and that some light is necessary for the clearing phenomenon. It is not safe to assume, however, that the great intensity of light at noontime is the cause of the frequent appearance of clearing of veins during hours near noon. There may be unknown factors affecting the time of first appearance of the clearing symptom during daylight hours. The usual appearance of uniformly bordered small veins in leaves showing clearing of veins is represented in Figure 6 A. A not uncommon phenomenon is represented in a similar leaf in Figure 6 B. In this leaf a few large secondary lesions are scattered over the apical portion of the area of cleared veins. Nearer the basal part of the leaf many tiny secondary lesions form an almost continuous border along small veins. The two sets of secondary lesions appear to differ in age, as though a few infections occurred first upon movement of virus from the inoculated leaf,

and a great many infections originated later. The lack of a completely graded series of lesions from largest to smallest suggests some factor other than the direct effect of sunlight in developing the clearing symptoms; in some way the time of day may be associated either with the movement of virus from the primary lesions of the inoculated leaf, or with the movement of virus from the tissues of the veins into intervenal tissues in the leaf showing secondary infection.

SPREAD OF VIRUS IN CUT AND UNCUT INOCULATED LEAVES

By the use of the iodine treatment described in the section on methods, starch retention in primary lesions in the inoculated area of a leaf can usually be detected about a day and a half after inoculation. The lesions are found to be progressively larger in specimens taken at successive intervals. After three or more days they are no longer all circular in outline, but some extend along large veins, soon bordering veins leading to the midrib (6, Fig. 2 C, E, F).

The area involved in the starch pattern stained by iodine is approximately that from which virus can be recovered at the time of collection of a specimen (6, p. 170; 12, p. 504). It is probable, however, that the conditions which interfere with the removal of starch arise somewhat later than the entrance of virus into previously normal portions of leaf tissue, and that starch retention patterns shown in iodine preparations are regularly a little smaller than the invaded areas. If the presence of virus interferes equally with the removal of starch in different tissues, it is probable that the discrepancy between the area marked by the starch retention pattern and the location of virus is small when the virus is invading new tissue slowly as in the expansion of primary lesions, and great when the virus is passing rapidly to distant parts of the infected plant. This seems to have been shown by earlier work (5, Tables IV and IX for example), in which virus was not found in parts of leaves comparatively near the inoculated area, but was shown to be present in quantity in some leaves at the top of the plant before the appearance of clearing of veins.

The uniformity with which movement of virus from the site of inoculation is associated with retention of starch along comparatively large veins just below or just above the point of infection and the rarity of retention of starch along small veins in the neighborhood suggested that virus did not generally utilize small veins in this movement. If small veins were not invaded, cuts through large veins in this region might possibly delay the infection of the remainder of the plant for a long time. It might be necessary for extensive spread of the primary lesion to take place before uncut large veins would be reached. The following experiments involving cutting of veins near the point of inoculation were therefore performed.

Six hundred plants of Turkish tobacco were grown in five sets of 120

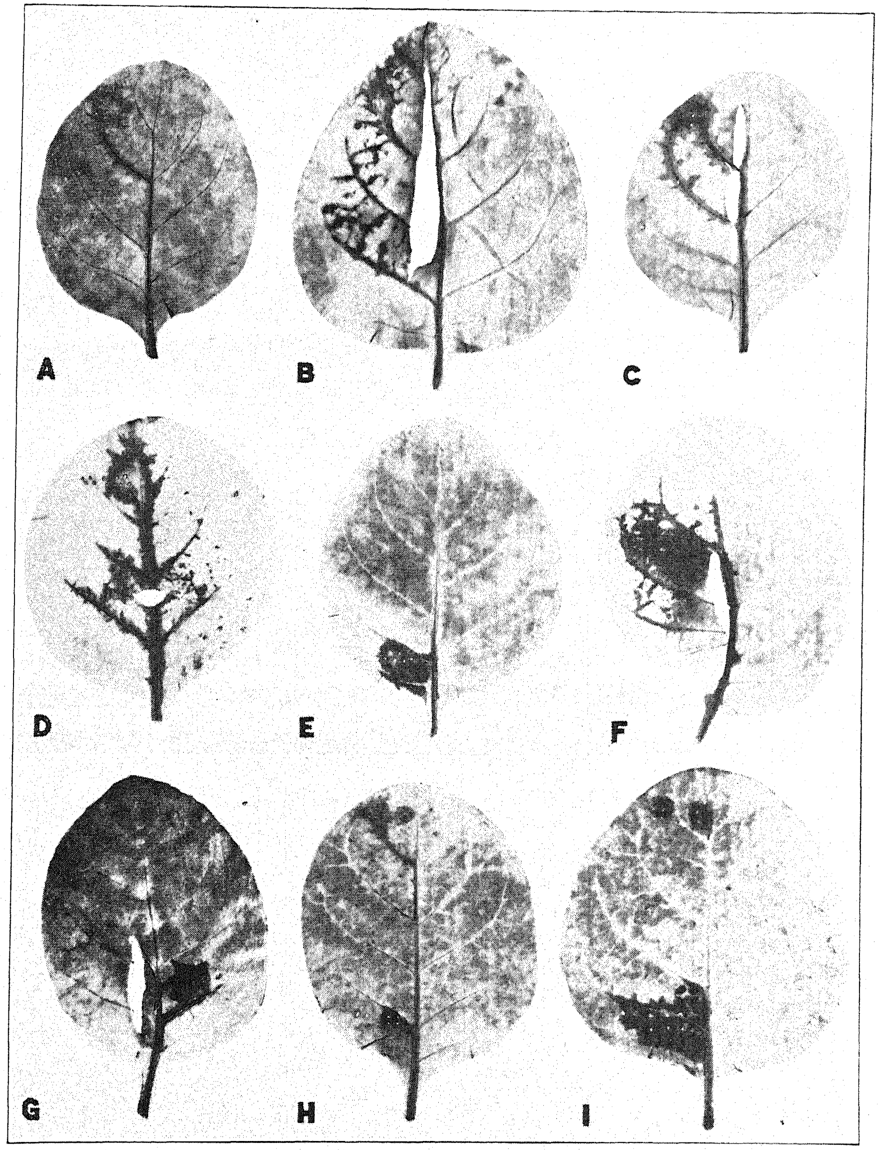

FIGURE 3. Leaves of *N. tabacum* var. Turkish, stained in iodine, showing typical patterns caused by virus of tobacco mosaic in spreading from cut and uncut leaves: A, Uncut leaf inoculated near apex; B, Veins and intervenal tissue cut; C, Intervenal tissue cut; D, Midvein cut; E, Uncut, leaf inoculated near base; F, Cut to base of leaf inoculated near base; G, Leaf inoculated on other side of midvein; H, Uncut leaf, small veins involved; I, Uncut leaf, pattern along midvein interrupted at points of entrance of side veins.

plants each. Each set of 120 was arranged in six groups of 20 plants each, so that all groups contained plants comparable in size and appearance. One set of six groups was used on each of the five occasions for the six types of inoculations described below. Inoculation was performed first in one plant of each group, then in a second plant of each, and then in successive plants of each group. In this way variations in inoculum, process of inoculation, and environmental conditions were distributed over all the groups and accidental variations in average results minimized.

The plants in the first four groups of each set were inoculated by ten pin punctures near the apex at one side of the midvein of the leaf. In the first group no cut was made in the leaf; in the second, a cut was made through both intervenal tissue and veins, between the point of inoculation and the midrib of the leaf; in the third, cuts were made through the intervenal tissues of the leaf without cutting the large veins; and in the fourth, a cut was made through the midrib between the point of inoculation and the base of the leaf. Such inoculation punctures and cuts may be seen in Figure 3 A to G. The plants of the fifth and sixth groups of each set were inoculated by ten pin punctures near the base at one side of the midvein of the leaf. In the fifth group no cut was made; in the sixth group a cut was made from the basal leaf margin to a point above the inoculation punctures.

Path of virus in uncut leaves. In the first series, inoculated near the apex of an uncut leaf, 53 plants became infected among 100 inoculated. The average interval between the time of inoculation and the time of appearance of clearing of veins in infected plants was 5.02 days. The variations in individual time of appearance of symptoms are shown in Table 1. In Figure 3 A a typical leaf from this series is represented as it appeared when collected and stained with iodine one day after the plant from which it was taken had shown systemic symptoms.

Path of virus modified by cutting side veins. In the second series, in which a cut was made through both intervenal tissue and veins, between the point of inoculation and midrib of the leaf, 42 plants became infected. The average interval between the time of inoculation and the time of appearance of systemic symptoms was 5.76 days. The delay beyond the time required for the first, or uncut control series amounted to 0.74 of a day. A day after the time of appearance of clearing of veins, marking the beginning of systemic infection, each plant was treated to show the starch retention pattern in the tissues through which virus had passed in the inoculated leaf. The stained specimens provided an explanation for the shortness of the delay. As shown in a photograph of a specimen in Figure 3 B, the virus was not confined under these changed conditions to passage through large veins as was the case in most of the uncut leaves, but appeared to have invaded innumerable small veins. These small veins were bordered by gray

TABLE I

DELAY IN AVERAGE TIME OF APPEARANCE OF SYSTEMIC SYMPTOMS IN PLANTS WITH INOCULATED LEAVES CUT IN VARIOUS WAYS

Series inoculated in various ways (100 plants in each series)	Number of plants beginning to show systemic symptoms on successive days — Time interval between inoculation and time of clearing of veins							Total diseased	Average expired time in days in each series	Delay in days in comparison with appropriate control series
	3 days	4 days	5 days	6 days	7 days	8 days	9 days			
Inoculated near apex:										
Not cut	1	23	12	9	7	1	0	53	5.02	—
Intervenal tissues and veins cut	1	5	14	10	7	5	0	42	5.76	0.74
Intervenal tissues cut	1	15	17	15	3	1	1	53	5.21	0.19
Midvein cut	1	13	14	7	7	3	1	46	5.41	0.39
Inoculated near base:										
Not cut	5	46	16	4	1	1	0	73	4.36	—
Cut from basal margin	4	25	27	10	2	3	2	73	4.97	0.61

bands from which starch had not been removed. The primary lesion normally covered an area including such small veins, but virus did not ordinarily escape into them, or, if it did, no indication of the occurrence was given by retention of starch along these veins. When the large veins no longer served to conduct the virus, because of cuts between point of inoculation and midrib, these smaller veins showed evidence of being invaded and of transporting virus to uncut large veins. The stained specimens showed that the number of intervenal areas in which small veins were invaded corresponded to the number of large veins which were cut. In general enough small veins were invaded to allow the virus to reach uncut large veins, giving access to the midrib and the remote parts of the plant; commonly no further extensive invasion of small veins took place. It may be questioned whether an extensive cut would cause the appearance of a starch retention pattern somewhat similar to that found in the case under discussion even in the absence of virus, since a cut more or less isolating an extensive leaf area might interfere with carbohydrate removal. It was found that uninfected leaves consistently removed starch so efficiently from the portions of the leaf partly isolated by such cuts as are described in this paper that no demonstration could be obtained to show that the cutting itself caused any abnormal starch retention during the period of storage in darkness. If any retention was caused by such cuts alone, it was not detected under the conditions of these experiments. It seems clear from this, as well as from the character of the pattern, that the observed retention was ascribable as in the uncut leaves to the effect of the presence of virus on the rate of disappearance of starch.

Path of virus in leaves with cuts in intervenal tissues. In the third series, in which cuts were made through intervenal tissues of the leaf, but not through large veins, 53 plants became infected. The delay was much less than in the series in which both intervenal tissues and veins had been cut, but there was a slight delay in development of systemic symptoms beyond the time required by plants with uncut leaves; there is a possibility that a few breaks which occurred in these veins after inoculation but before the time of appearance of symptoms of the systemic infection may have contributed in part to this slight delay. The average interval between the time of inoculation and the time of appearance of systemic symptoms was 5.21 days, 0.19 of a day more than for the uncut plants inoculated in the same way. Stained specimens showed that the cuts in the intervenal tissues generally interfered little, and sometimes apparently none, with the normal pattern. In Figure 3 C a typical example of this series is presented. The failure of cuts in intervenal tissues to delay the movement of virus to the top of the plant as much as a cut through large veins, seemed to support the idea that the intervenal tissues were less important in the rapid systemic spread of virus than were the large veins. This idea was also sug-

gested by the failure of these cuts to interfere much with the path of virus through the leaf tissues.

Path of virus modified by cutting midvein. In seemed probable that if the midvein of an inoculated leaf were cut, the virus would pass through leaf tissues around the cut to reach points on the midrib beyond the obstruction; but there seemed to be no basis for prediction of what area of leaf tissue would be affected.

In the fourth series, in which a cut was made through the midrib, 46 plants became infected. The appearance of symptoms of the systemic infection was slightly delayed by the midrib cuts, the average interval between the time of inoculation and the time of appearance of systemic symptoms being 5.41 days, or 0.39 of a day longer than for the similarly inoculated uncut plants. The stained leaves showed that a small portion of the leaf area, near the cut, was invaded by the virus in its passage around the incision. A typical example of the pattern around a cut in the midrib is shown in Figure 3 D.

Path of virus modified by cutting to basal margin. It had been noted (6, p. 166) that the normal starch retention pattern extended rapidly toward the base of the leaf, but slowly toward the apex of the leaf. It was therefore of interest to determine the effect of a cut from the basal margin toward the apex of the inoculated leaf on the time required for movement of virus from an inoculated area near the leaf base.

In the fifth or control series in which each plant was inoculated near the base of an uncut leaf, 73 plants became infected. The average time required for this control series was 4.36 days. In the sixth series, in which a cut from the basal margin of the inoculated leaf was made parallel to the midvein and between the midvein and the inoculation point near the base of the leaf, 73 plants became infected. The average time required for this sixth series was 4.97 days. The delay therefore amounted to 0.61 of a day. Typical specimens of the fifth and sixth series are represented in Figure 3 E and F. In Figure 3 G is represented a leaf in which inoculation punctures were made on the side of the leaf opposite the cut; it will be noted that the cut itself did not cause starch retention in the partly isolated region.

INOCULATION NEAR BASE AND APEX OF LEAF

Ease of infection. In the above-described experiment the two control series differed in two ways; systemic symptoms occurred earlier in the series inoculated near a leaf base, and many more infections occurred in the plants of this series than in the similar plants inoculated near a leaf apex. In the latter respect both cut and uncut series were similar. The proportions of infection in the two series inoculated at a leaf base were 73 per cent in both cases; in the four series inoculated simultaneously near a leaf apex, however, the proportions were 53, 42, 53, and 46 per cent.

This suggested that tissues near a leaf base were more easily infected by pin puncture inoculation than tissues near a leaf apex.

An experiment was conducted to compare the effectiveness of basal and apical inoculations when both types were made in the same leaf. On two occasions sets of 100 plants each were inoculated with ten pin punctures near the apex and ten pin punctures near the base of one leaf of each plant. All infected plants were segregated when they showed clearing of veins. The plants were allowed to remain in the greenhouse one day after the symptoms of the systemic infection had been seen, and were then removed to dark rooms to prepare leaves for staining to show the number of primary lesions on the inoculated leaves. Of the 200 plants, 185 became infected; they showed 177 lesions as a result of the 2000 apical inoculation punctures, and 324 lesions as a result of the 2000 basal inoculation punctures.

In this and in the preceding experiment more infections arose from inoculation near the leaf base than from similar inoculation near the leaf apex. The results of these experiments furnish evidence that the leaves of young Turkish tobacco plants are more easily infected by pin puncture inoculation at leaf base than by similar inoculation at leaf apex.

Relative susceptibility of young and old leaf tissue. Although not related to the experiments above described, a test of very young leaves may be cited here to indicate that younger leaves of the plant are more easily infected by pin puncture inoculation than are any of the tissues of nearly expanded leaves previously discussed. In an experiment conducted with very young leaves, a leaf near the growing tip of each of a number of plants was inoculated with a single pin puncture. In 215 plants inoculated in this manner, one primary lesion was produced for each 1.94 pin punctures. It is believed that such a high degree of relative susceptibility to pin puncture inoculation has never before been shown for this or any other virus disease of plants. It is interesting to compare this incidence of one primary lesion for each 1.94 pin punctures in very young leaves, with the results from an experiment cited above which amounted to one lesion for each 6.2 punctures in the case of 2000 punctures in the basal portion of the more fully expanded leaves, and one lesion for each 11.3 punctures among 2000 punctures near the apices of these same nearly expanded leaves.

Time of appearance of systemic symptoms. In the experiment in which there were two control series, the plants of one inoculated near a leaf base and those of another near a leaf apex, the series inoculated near the base of an uncut leaf showed systemic symptoms much earlier on the average than did the series inoculated near the apex. The average time between inoculation and the appearance of systemic symptoms was 5.02 days in the apically inoculated series, but 4.36 days in the basally inoculated control series. The difference in time amounts to 0.66 of a day. Some difference

might be expected because of the shorter distance to the top of the plant from the base than from the apex of the inoculated leaf. Whether this factor is important is a question which can be answered better after consideration of the possible effect of the relative susceptibilities to infection of the tissues at leaf base and at leaf apex.

It appeared probable that the earlier spread from basal inoculation was caused in part by the greater numbers of primary lesions present. For this reason it was necessary to perform another experiment, in which this factor would be eliminated, to determine whether or not an independent difference in earliness of systemic spread of virus could be detected. Two hundred plants were divided into two comparable sets of 100 plants each, and one set was inoculated by five punctures near the base of a leaf of each plant, the other set by ten punctures near the apex of a similar leaf. It was believed from former experience that five punctures near the base of a leaf would result in about the same average number of lesions as would be caused by ten punctures near the apex of a similar leaf in each plant. All plants were isolated and prepared for staining in iodine as soon as they showed clearing of veins. When the set had been treated it was found that the plants inoculated at leaf base had furnished 69 systemic infections, as a result of 122 primary lesions. The plants inoculated at leaf apex had furnished 88 systemic infections, as a result of 159 primary lesions. The average time between inoculation and the appearance of systemic symptoms, however, had been 4.95 days in the basally inoculated set, and 5.28 days in the apically inoculated set. From this result it appears that the virus reached the upper leaves of inoculated plants more quickly from basal inoculation than from apical inoculation, in spite of slightly more infections in the plants with apical inoculation. The experiment indicates that the greater susceptibility of the tissues near the base of a leaf did not account altogether for the earlier systemic symptoms in plants inoculated near a leaf base than in plants inoculated near a leaf apex. It suggests that virus leaves the site of inoculation earlier when plants are infected near the base of a leaf than when similar plants are infected near the apex of a corresponding leaf, although the possible effect of the shorter total distance to top of plant from base than from apex of leaf is still to be considered.

RELATION OF EXPANSION OF INOCULATED LEAF TO MOVEMENT OF VIRUS

Inoculated leaf not covered. Since the time required for spread of virus from the base of a leaf was less than that required for spread from the apex, an explanation for the difference was sought.

Casual observation had shown that inoculation punctures spread apart gradually between the time of inoculation and the time of appearance of symptoms, because of growth during this period. A series of measurements taken during the first ten days after inoculation of a set of plants showed

that inoculation punctures in tissues near the base of the leaf spread apart more rapidly than those in tissues near the apex. This observation led to the hypothesis that the earlier spread of virus from the basal than from the apical inoculation was correlated with the greater ability for expansion in the basal region of the leaf at the time of inoculation.

It appeared probable that inoculated leaves, although chosen as comparable, would generally differ slightly in degree of maturity and rate of expansion. Upon trial this was found to be true. Some individual leaves showed considerably greater lateral expansion than others.

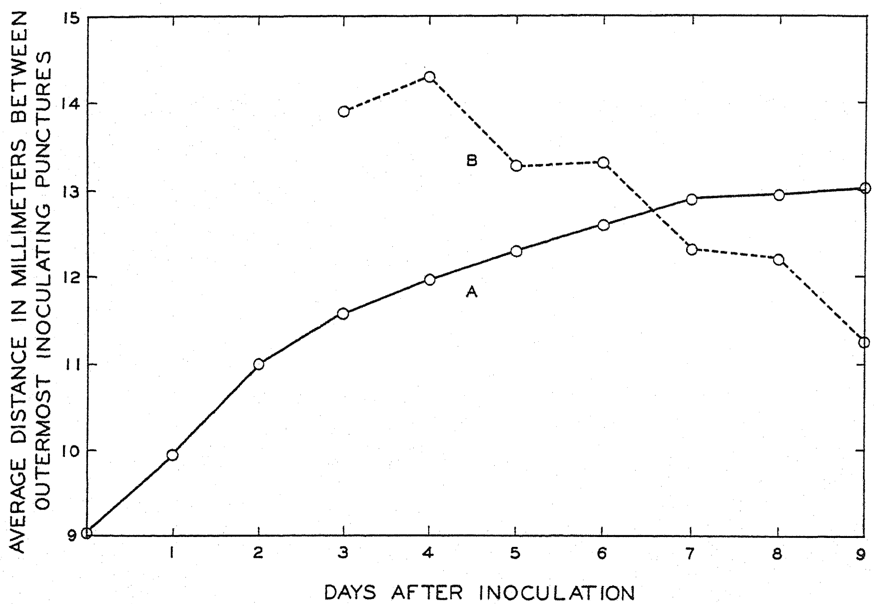

FIGURE 4. Comparison of degree of expansion of inoculated leaves of *N. tabacum* var. Turkish on successive days after inoculation by pin punctures: A, Average of all control plants; B, Averages for sets showing clearing of veins on each day.

In order to test the hypothesis that early spread of virus was correlated with ability to expand rapidly in the inoculated area, a set of 120 plants was inoculated as uniformly as possible by means of 20 punctures in one leaf of each plant. Twenty of the plants were used as controls, being measured daily to secure a record representing average expansion of inoculated leaves. The remaining 100 plants were not measured for expansion of the inoculated areas from day to day, since in making measurements contaminations might have occurred, but from these plants groups were selected as showing clearing of veins on successive days. These groups were examined on the day of appearance of clearing of veins, and records were made of distance between extreme edges of inoculation punctures. In

Figure 4 the averages of daily measurements of distance between inoculation punctures on all the control plants are indicated by an unbroken line, and in the same figure the average measurements for sets of plants showing systemic symptoms on successive days are represented by points on a dotted line. It is evident from the opposite trends of the two lines that the plants which showed symptoms early were those of which the inoculated leaves had expanded rapidly since inoculation, and that plants showing symptoms later were those of which the inoculated leaves had expanded much more slowly. The plants which showed systemic infections earliest had expanded in the inoculated region in a very short space of time by more than 50 per cent of the width at the time of inoculation, an expansion greater than the average of the measured control set ever attained. The plants which were latest to show systemic symptoms had expanded in the inoculated region by less than 25 per cent of the original width, much less than the average of the control set at the time. It was shown by this experiment that the ability of the inoculated leaf to expand, an easily determinable character, was correlated under the conditions of this experiment with time of spread of virus from primary lesions, but it was not shown that a causal relation existed. This experiment, in which inoculation punctures were located in all leaves at approximately the same position, indicated that in earlier experiments the quicker development of the systemic disease from infection near leaf base than from equal infection near leaf apeq probably depended not so much on the slightly shorter path to the top of the plant as on the character of the tissues near the leaf base. The leaf base tissues are young and capable of rapid expansion, and conceivably may allow easier movement of virus.

The phenomenon of association of early systemic infection with inoculation of relatively young and rapidly expanding leaf tissue may throw light on the course of events in the systemic phase of the disease when the virus returns into intervenal leaf tissues in young leaves of the inoculated plant. Clearing of veins at the bases of young leaves occurs very soon after the time when virus can first be demonstrated in the midvein or petiole of the inoculated leaf. The tissues thus involved constitute the younger portion of the affected leaves. Involvement of the older apical portions of these leaves is comparatively slow. Movement of virus into old leaves also occurs, but it is extremely slow. The nature of the changes in cells with increasing age, making tissues progressively less readily permeated by virus, is worthy of study in the effort to learn how to control or modify the spread of virus in the plant. The virus must have some property, or properties, which allows it to move from a cell of one tissue to a cell of another tissue, yet subjects it to delay. This is a matter of great interest in relation to the mechanism by which virus spreads through the plant, and in relation to the ultimate nature of the virus.

Inoculated leaf covered. The correlation between ability of a leaf to expand rapidly and the property of allowing virus to spread early from the area of inoculation to distant parts of the plant appeared upon further tests not to apply when expansion of the leaf was arrested by covering the leaf. It was found that if a leaf was covered with tin foil during the latter part of its period of active expansion the increase in width of a marked area almost stopped within 36 hours. This loosely applied tin foil excluded light, and of course interfered slightly with movement of gases. If such a leaf was later uncovered expansion might become rapid again. This is represented by a graph in Figure 5.

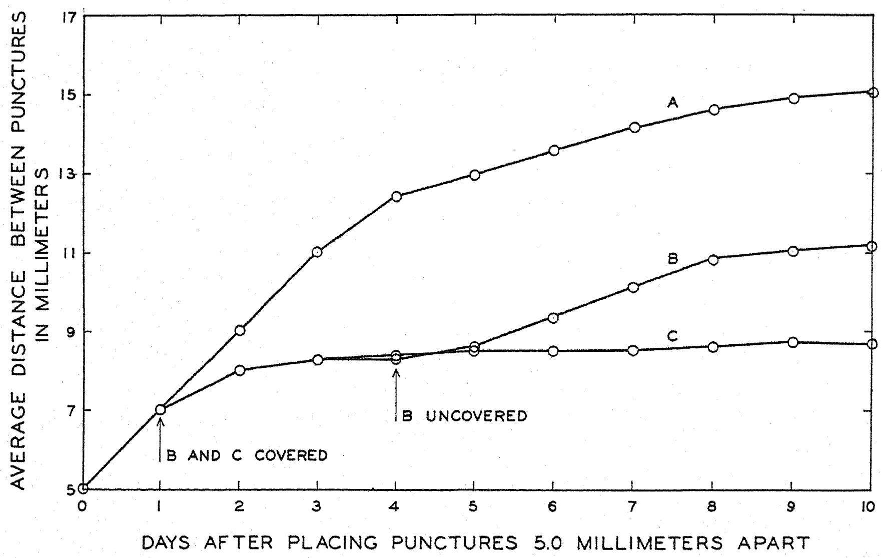

FIGURE 5. Expansion of growing leaves of *N. tabacum* var. Turkish: A, Unshaded leaf; B, Leaf wrapped in tin foil 24 hours after observations started; exposed to light again three days later; C, Leaf wrapped in tin foil 24 hours after observations started; covered continuously except during brief daily examination at 5 p.m.

Since early movement to initiate the systemic disease had been so markedly correlated with rapidity of growth of the inoculated leaf in former experiments, it was naturally thought that considerable delay in appearance of the systemic disease might occur as a result of covering the inoculated leaf immediately after inoculation. Several sets, each consisting of 40 Turkish tobacco plants, were heavily inoculated on one leaf of each plant and subsequently were treated in different ways. Two of the sets served to give preliminary information in the question under discussion. In one of them the inoculated leaves were wrapped in sheets of tin foil immediately after inoculation; in the other the inoculated leaves were not

covered. The plants of the control set, in which the inoculated leaves were not covered, all showed clearing of veins within the daylight hours of the fifth day after inoculation. The plants with inoculated leaves covered with tin foil showed little expansion of the inoculated leaves after the time of inoculation, but they showed less than a day of delay in appearance of clearing of veins. This result, which was confirmed consistently in later experiments, indicated that the cessation of expansion of leaves darkened for a few days did not greatly delay virus movement. The cessation of expansion in leaves covered with tin foil must differ essentially from the partial slowing of expansion in the maturing leaf. One of these processes may perhaps be associated with destructive processes following the local starvation with respect to carbohydrates; the other may be the result of constructive processes in the formation of the cell as it approaches maturity.

MODIFICATION OF PATTERNS OF CLEARING OF VEINS

Production of unsymmetrical patterns. In experiments described earlier in this paper it was shown that radical operations, such as extensive cutting of inoculated leaves, caused the virus to move with slight delay over paths not ordinarily taken in leaving the inoculated leaf. In cut leaves small veins of the leaf were utilized in the movement of virus around the cuts, although such veins did not ordinarily give evidence of being involved in uncut leaves. It was shown also that certain other changes in plants, such as increased age, modified the rate of movement of virus from the inoculated leaf.

In the experiment just described, the rate of movement of virus was not much modified, but a striking change of the path affected by the virus in the plant was secured by shading the inoculated leaf. A great many one-sided infections of the inoculated plants appeared, and a large proportion of the leaves showing clearing of veins also showed one-sided infection as manifested by unsymmetrical patterns similar to the one represented in Figure 6 C. This result suggested the desirability of shading various leaves of inoculated plants. Experiments conducted to study the effects of shading leaves other than the inoculated one brought to light the curious fact that the path of the virus in the systemic infection could be modified and to some extent controlled both by shading inoculated leaves and by shading leaves which were not inoculated and which did not have virus in them during the course of the experiment.

The first experiment with covered inoculated leaves was repeated. A far larger proportion of all the leaves showing clearing of veins in the set with inoculated leaf covered with tin foil showed unsymmetrical patterns than of the leaves in the control set in which the inoculated leaf was not covered. The total number of such patterns was also greater in the set with inoculated leaf covered with tin foil, in spite of the fact that the plants with

covered inoculated leaves showed clearing of veins on fewer leaves of each plant. The smaller number of leaves affected by clearing of veins on plants with covered inoculated leaves appeared to be due to absence of clearing of veins from entire leaves developing on the side of the stem opposite that to which the inoculated leaf was attached. Since the plants in this second leaf-covering experiment showed a somewhat larger number of leaves with one-sided infection in the control set than had been observed in the first experiment, and since the nature of the experiment indicated the possibility that this might have resulted from accidental shading of inoculated leaves in the control set, it was decided to repeat the experiment.

A set of 120 plants was inoculated on one leaf of each plant, and the set was then subdivided. In one-third of the set, complete shading of the inoculated leaf was ensured by covering it with tin foil, but the remainder of the mature or nearly mature leaves of the plant were left exposed to light. In another third of the set, the inoculated leaf was left uncovered, but two mature or nearly mature leaves near the level of the inoculated leaf but on the other side of the stem were covered with tin foil. In the remaining third of the set no leaves were covered.

The plants with covered inoculated leaves showed 84 leaves with clearing of veins, and of these 48 showed unsymmetrical patterns. The plants with two covered uninoculated leaves showed 109 leaves with clearing of veins, but none of these showed such patterns. The plants with no covered leaves developed clearing of veins on 117 leaves; of these 13 showed one-sided infections. This experiment gives evidence that the distribution of virus to the top of the plant can be modified by shading appropriate portions of leaf surface in the lower part of the plant.

Production of patterns extending toward periphery. Another variation in the clearing of veins pattern was produced by inoculating comparatively young leaves. As has been indicated in the previous parts of this paper, whenever half-expanded or larger leaves were inoculated in such a way that they developed one or a few primary lesions, it could be seen clearly by staining with iodine soon after the appearance of systemic symptoms in the top of the plant that the virus had affected a small, more or less circular portion of tissue around the point of inoculation, and had also affected a path of tissue along the nearest large vein and the midvein. Small veins were not usually involved in the inoculated leaf. Occasionally, however, inoculation near the base of a leaf allowed the formation of a small area of cleared veins nearby, although similar inoculation near the apex of the same leaf did not produce such an effect. It seemed probable that the inoculation at the base was in tissue so young as to respond differently from the tissue surrounding the point of inoculation near the older leaf apex. Occasionally, also, inoculation near the apex of a young leaf resulted in development of a clearing pattern, not near the point of inoculation, but

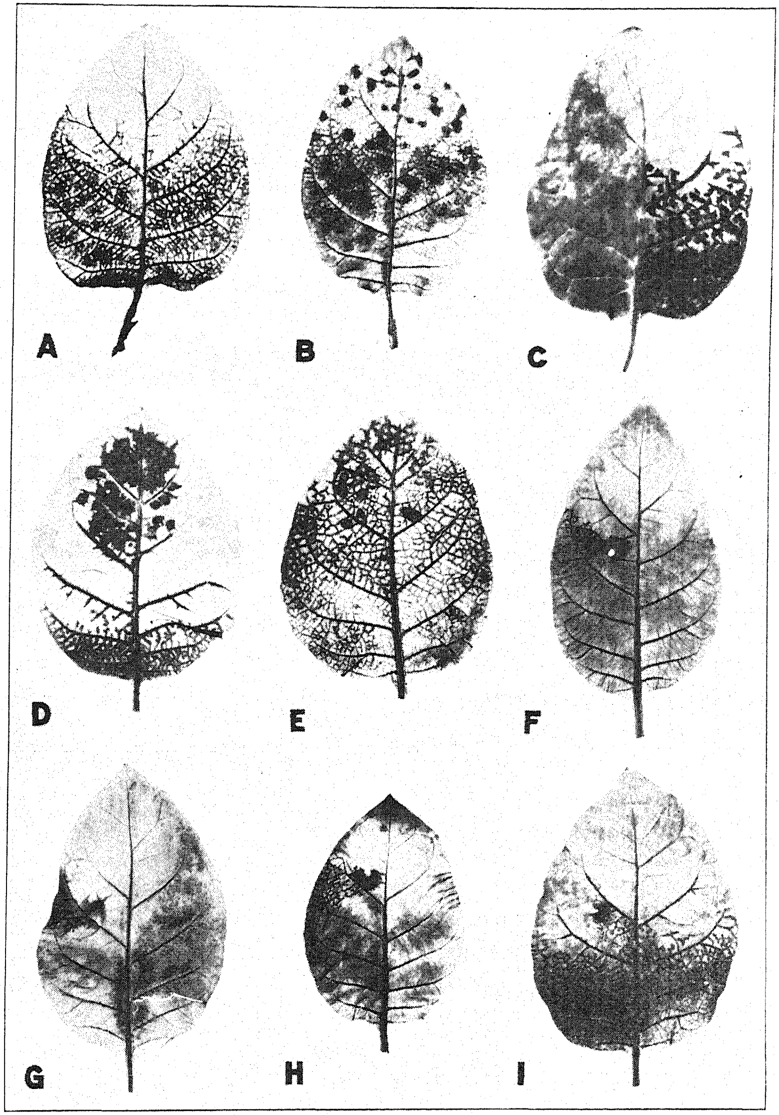

FIGURE 6. Starch retention patterns corresponding to patterns of clearing of veins in leaves of *N. tabacum* var. Turkish stained in iodine: A, Typical pattern; B, Large spots as well as narrow border on small veins; C, One-sided pattern produced by shading inoculated leaf; D, Leaf typical of set inoculated near the apex when $1\frac{1}{2}$ to $2\frac{1}{4}$ inches long; E, Leaf typical of set inoculated near apex when $\frac{3}{4}$ to 1 inch long; F, G, H, I, Leaves representative of set inoculated by single pin punctures in leaves less than $\frac{1}{2}$ inch long.

at the base of the inoculated leaf. It seemed probable that this phenomenon was the result of the inoculation of an unusually young leaf. This hypothesis was confirmed by inoculating series of young leaves of different sizes. It was found that when leaves less than two inches long on rapidly growing plants (three inches high in four-inch pots) were inoculated at their tips, clearing of veins commonly occurred at the base of the inoculated leaves (Fig. 6 D); and further, that if the inoculated leaves were less than one inch long a great part of the area of the leaf, even including the point of inoculation, might be involved in the clearing of veins pattern (Fig. 6 E). This suggested the possibility of testing, by inoculation into an even smaller leaf, whether a primary lesion in a young leaf would extend along veins mainly toward the base, as does the ordinary primary lesion of the disease in an older leaf, or peripherally as clearing of veins patterns extend from the base of young leaves.

Young leaves, one-half inch or less in length, at the center of rapidly growing plants of Turkish tobacco were inoculated by means of single punctures with No. 00 insect pins previously used to puncture yellow areas of well-mottled mosaic leaves. The inoculated leaves grew rapidly and on the sixth day after inoculation showed a variety of patterns, almost all of which included clearing of veins near the point of inoculation. In some cases the clearing involved almost all the veins below the point of inoculation (Fig. 6 I), in others only a part of the veins were involved (Fig. 6 H); in many cases an area between the point of inoculation and the periphery of the leaf was involved (Fig. 6 F), sometimes with less strongly marked and presumably later initiated clearing of veins toward the base of the leaf. The yellowish patterns in the living leaf were nearly as clear as the starch retention patterns in the stained leaf. It appeared that in some young leaves an early movement of virus toward the leaf periphery was succeeded by a movement toward the leaf base.

In some of the youngest leaves inoculated by means of single punctures the area between the point of inoculation and the nearest part of the leaf periphery was invaded by virus, not with the production of narrow bands of affected tissue along small veins, but with formation of a solid yellow area in the living leaf. These areas appeared in the stained leaf as similar solid areas in which starch was retained abnormally long upon incubation in darkness. The yellow area in the living leaf looked like the yellow area of a mottled mosaic leaf. Like the areas of yellow in the first mottled leaves of the ordinary systemic infection, the yellow area thus produced at the point of inoculation was abnormal in expansion, and so the affected leaf presented the phenomenon of distortion of contour in the region of the yellow area (Fig. 6 G). This production of a yellow area associated with distortion of the contour of the leaf, suggests that in the ordinary systemic infection the varied phenomena of clearing of veins, mottling, and distortion are determined by the age of each leaf when invaded by virus.

Early production of clearing of veins in partially defoliated plants. In *Nicandra physalodes*, as reported in another paper (7), it was found possible to hasten the appearance of systemic infection by partial defoliation. Tobacco mosaic virus multiplies rapidly in inoculated leaves of this host, but frequently does not become systemic in plants inoculated after the early seedling stages of development. Removal of all leaves except the one inoculated and the young leaves near the growing points was followed by the appearance of yellow spots which characterize the systemic infection in this plant.

Tobacco mosaic virus is not so conspicuously localized in *N. tabacum*, but in old plants there is a considerable interval between inoculation and appearance of clearing of veins at the time of onset of systemic infection. Thirty plants of *N. tabacum*, each about nine inches high, were inoculated by rubbing infectious juice over a leaf half way up the stem. Alternate plants were then defoliated as the *N. physalodes* plants had been, leaving the inoculated leaf and the very small leaves near the growing point. On three occasions during the first six days after inoculation, the larger top leaves were removed. Clearing of veins occurred in 3 of the 15 partially defoliated plants on the fifth day after inoculation, and in 11 of the 15 before any of the control set had shown systemic symptoms. Systemic symptoms appeared in all control plants within nine to nineteen days after inoculation. The corresponding period for the appearance of systemic symptoms in the partially defoliated plants was five to nine days. It appears therefore that systemic infection may be hastened by partial defoliation.

UNCOMMON PATHS OF VIRUS IN CUT AND UNCUT LEAVES

In the cut and uncut series of leaves discussed in a previous section of this paper, occasional patterns in inoculated leaves differed from the common types of patterns in their sets. As mentioned above, clearing of veins sometimes occurred in the basal portion of the inoculated leaf. Two other unusual patterns are represented in Figure 3 H and I, because they are suggestive of occasional variations in resistance to movement of virus in supposedly normal leaf tissue.

In Figure 3 H a pattern from one of two apical lesions on a leaf shows evidence of interference with movement of virus in the midvein. The evidence is given by abrupt termination of the path affected by the presence of virus, and by the presence of a series of secondary lesions along small veins. These small veins extend to a large vein which connects with the midrib below the point of interference. Tissues are affected along both sides of the side vein leading from the inoculated area, but on only one side of the midvein. In most leaves the midvein shows an affected area on both sides. Such one-sided infection of the midvein perhaps accounts for the observation that some cuts in the midvein of leaves have resulted in

invasion of the neighboring tissues on the side of the midvein nearer the primary lesion, but not on the other side.

In this leaf the path taken by the virus through small veins is marked by a series of secondary lesions of small size. This suggests that the bordering of veins leading from inoculated areas may be caused by the formation of innumerable secondary lesions along these veins during the movement of virus through them. When only a few spots appear, as in Figure 3 H, evidence is supplied that virus can move through veins without visible effect, and that virus affects tissues only where it sets up secondary centers of multiplication.

In Figure 3 I is represented a similar leaf in which the path from one of two apical lesions leads down the midvein a short distance and then stops abruptly. The affected area is bounded on one side of the midvein by a side vein, and on the other side of the midvein at another level by another side vein. It may be that the presence of the side vein was in some way connected with the obstruction which apparently limited the pattern of starch retention along the midvein.

DISCUSSION

INTERVAL OF TIME BETWEEN INOCULATION AND APPEARANCE OF SYSTEMIC SYMPTOMS

In the literature concerned with infections of tobacco mosaic virus in *N. tabacum*, reports of the time required for the production of systemic symptoms have shown considerable variation. E. M. Johnson (8) recently reported intervals of 3 to 15 days, whereas Klebahn (9) recorded intervals of 16 to 140 days. These intervals have sometimes been recorded as characteristics of virus samples. Johnson (8, p. 294) reported that one of two strains of severe mosaic of tobacco obtained in field collections had an incubation period about the same as the other, but symptoms might be visible a day sooner. In the same paper (8, p. 293–294) he stated that systemic symptoms of tobacco mosaic appeared 3 to 15 days after inoculation from either fresh or dried material, the average incubation period being 2 to 5 days longer with extract from dried material than with that from fresh tissue.

It is shown in the present paper that the average interval between time of inoculation and appearance of systemic symptoms is shorter when the number of primary lesions is large than when the number of primary lesions is small; and when the inoculation is near the base of a leaf than when it is near the apex of a leaf. Previously (5) it was shown that inoculation of a young leaf results in a much shorter interval before appearance of systemic symptoms than inoculation of an old leaf of the same plant. These considerations suggest the need for quantitative studies of any instances in which such intervals seem to be characteristic of virus samples, and in-

dicate that attention must be given to securing strictly comparable types of inoculation in each determination.

It seems worth while to call to the attention of those working with tobacco mosaic virus the possibility of shortening experiments by using inoculation methods best suited to producing prompt systemic infection in all cases in which the severity of inoculation and the age of the inoculated tissue are not otherwise determined by the nature of the experiments.

SPREAD OF VIRUS THROUGH LARGE AND SMALL VEINS

The extension of primary lesions along large veins in uncut inoculated leaves appeared to indicate that large veins were important for movement of virus from the area of inoculation. The interference with the shape of the pattern in plants in which a cut was made through large veins between the area of inoculation and the petiole of the inoculated leaf seemed to confirm this. The slight but significant delay in appearance of systemic symptoms in such plants, and the failure of cuts involving only intervenal tissues to produce similar results gave further confirmation.

Primary lesions in uncut leaves generally did not form extensions along small veins, although in enlarging they reached such small veins in the tissues of the leaf before they touched any large veins. This suggested that small veins were more difficult to invade than larger ones. Nevertheless, whenever large veins in the path of the virus were cut, small veins showed evidence of being invaded over extensive areas.

The affected areas around cuts appeared more extensive than the affected areas in uncut leaves examined after the same intervals following inoculation. It appeared probable, therefore, that the virus did not wait until the normal increase in size of the primary lesion carried it past the obstructing cuts, but that it was carried rapidly around the obstructions, as though by some diverted stream, through small veins and into parts of the leaf which were not ordinarily so promptly invaded. As a result, systemic infection was produced only a little later than in plants inoculated in uncut leaves. Allard (1) made similar experiments with cuts and found no conspicuous delay of virus movement to the top of the infected plant. The present experiments agree with his, and furnish some explanation of the shortness of delay.

In none of the cut leaves did severe wilting of partly isolated areas occur, even after many days. It was therefore evident that water was able to move around the cuts to the most distant portions of the leaves. The failure of the area of infection to extend rapidly in the direction of this water movement and its abnormally rapid extension in an opposite direction agreed with Caldwell's demonstration (3, 4) that the distribution of virus is not normally in the xylem fluids. The fact that the virus extended toward the leaf peripheries very slowly in all inoculated old leaves, as men-

tioned also in an earlier publication (6), seemed to be somewhat at variance with the finding that the virus moved both upward and downward in the stem very rapidly (4, p. 293; 5, p. 574). This might be explained, however, on the basis of movement with food substances to dependent parts of the plant.

The experiments on the production of many one-sided infections in leaves showing clearing of veins by covering the inoculated leaf on each plant, and on the elimination of such patterns by exposing the inoculated leaf to sunlight and covering leaves on the other side of the plant suggest the possibility that the virus may be carried with exports of food material from leaves, or by the mechanism ordinarily serving to transport such material. Covering inoculated leaves decreased the total number of leaves showing clearing of veins, but increased the number of leaves showing unsymmetrical infections. It seems possible that covering inoculated leaves may have induced some limitation of clearing of veins to one side of the plant from failure of the covered inoculated leaf to supply much food to the growing tip leaves. Movement of excess food material from the opposite side of the stem to the side bearing the inoculated leaf may have restrained virus movement from the sector bearing the inoculated leaf. Covering uninoculated leaves opposite the inoculated leaf eliminated unsymmetrical infections of leaves showing clearing of veins in the experiment reported. The procedure may have induced complete or nearly complete distribution of food substances from the inoculated leaf, incidentally distributing virus to all leaves of the top of the plant. In plants with leaves not shaded with tin foil, some unsymmetrical infections occurred. Shading of one leaf by another may have caused occasional disturbances in the balance of food materials on different sides of the plant.

If movement of virus with a particular food material could be proved, the virus would serve as a useful indicator of the distribution of this substance, since the virus is foreign to the normal plant, can be introduced at a selected point, and marks the path it takes in leaves involved in its distribution. The change in the path of the virus, resulting from covering either inoculated leaves or leaves neither inoculated nor at any time containing virus, suggests some direct or indirect connection with the carbohydrate supply, but the ability of the virus to move quickly from a leaf starved with respect to carbohydrates suggests that the relation may be indirect. The reversal of the direction of movement of virus in leaves inoculated when very young seems to indicate a relation of some kind between movement of food into a young and dependent leaf and the movement of virus, and suggests that virus moves toward the periphery until the leaf reaches a degree of maturity which allows it to export some food material to dependent growing parts.

The earlier spread of virus to the top of an old plant when all leaves

but the one inoculated and young leaves near the growing points were removed suggests that the inoculated leaf under these circumstances may supply more food to the dependent leaves at the top than it does when younger but expanded leaves are allowed to remain on the plant, and that virus may go with this food or be influenced in its spread by changed conditions in the tissues.

DELAYS IN MOVEMENT OF VIRUS

It has been the common opinion that *Nicotiana tabacum* is both highly susceptible to and easily permeated by the virus of tobacco mosaic. It seems, however, that a certain amount of evidence is now at hand tending to suggest the existence of some interference with free and uniform movement of virus within the tissues of the leaf.

The evidence seems insufficient to determine whether the interference with virus movement is of the nature of interference with customary speed of movement of virus within cells, as by decrease of protoplasmic streaming, or by decrease of translocation of food materials; or of the nature of interference with movement of virus from one cell to another, as from some restriction of the passage through cell walls, or from some modification of the permeability of cell membranes.

Further study of this interference with virus movement, the presence of which in various degrees of efficiency in different parts of the same plant of *N. tabacum* has been indicated in this paper, may throw light on several problems. It may help to explain the nature of hosts of tobacco mosaic virus which localize the virus conspicuously at ordinary temperatures, such as *N. glutinosa* L. and *Datura stramonium* L. It may lead to a better understanding of the nature of certain symptomless carriers such as *N. glauca* R. Grah., in which there seems to exist a tendency to delay and minimize escape of virus to the tops of plants from inoculated or subsequently invaded leaves in which the virus may be multiplying rapidly. It may further furnish information concerning some phase of the nature of the virus itself, since the virus appears to possess a property so adjusted to the character of the cells of the plant in which it is propagated that the virus can pass from cell to cell, but meets opposition and a measure of delay in this passage.

SUMMARY

A study was made of the movement of virus from the inoculated leaf in *Nicotiana tabacum* var. Turkish infected with tobacco mosaic virus.

The interval of time between inoculation and appearance of first systemic symptoms, clearing of veins, proved to be dependent on the number of primary lesions resulting from inoculation.

In old plants, movement of virus to the top was earlier when all expanded leaves except the one inoculated were removed than when all leaves were allowed to remain on the plant.

Tissue near the base of the leaf appeared more easily infected by pin puncture inoculation, and more capable of allowing early movement of virus from the leaf than tissue near the apex.

First indications of clearing of veins appeared commonly during hours near noon, and rarely during hours in late afternoon, night, and early morning.

The number of one-sided infections in leaves showing clearing of veins was increased by shading the inoculated leaf in each plant, and reduced by shading leaves on opposite side of the stem.

Clearing of veins from the point of inoculation toward the periphery occurred in leaves inoculated while young, and was succeeded by clearing of veins toward the base of the inoculated leaf. Inoculation of younger leaves produced yellow areas showing irregular expansion, resembling the yellow areas of leaves showing mottling and distortion in the systemic infection with tobacco mosaic.

In plants in which a cut was made through large veins between the area of inoculation and the petiole of inoculated leaves, brief delays in movement of virus to the top of the plant were noted. Associated with these delays were marked modifications of the patterns formed by the influence of virus upon retention of starch in recently invaded portions of the inoculated leaf. Plants in which cuts were made in intervenal tissues but not through large veins showed little delay.

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SYMPTOMS OF TOBACCO MOSAIC DISEASE

Francis O. Holmes

INTRODUCTION

The literature concerned with the symptoms produced in various species of plants in response to infection with tobacco mosaic virus deals chiefly with mottling, masked, and necrotic effects. Mottling symptoms are best known, and have been considered the typical expression of the presence of this virus in its hosts.

The object of the present paper is to describe some variations of mottling, masked, and necrotic symptoms, and certain previously undescribed effects, such as prolonged yellowing, leaf abscission, outgrowth of tissue on the lower side of mottled leaves, bending of upper stem toward side of plant bearing inoculated leaf, and intensification of pigment in spots on flowers.

MATERIALS AND METHODS

The virus used in this study was the widely-used field type of tobacco mosaic virus, closely resembling and perhaps identical with the virus used by Iwanowski (9), Allard (1), and Goldstein (4), and with Johnson's (10) tobacco virus 1. The relation of the virus used in this work to strains used by others may be judged by the reactions described for the host plants discussed in this paper.

Observations were made on all species of plants used in this study under greenhouse conditions, where contaminating viruses could be excluded. Observations were made also on nearly all of these species under outdoor conditions in the Institute garden. The presence of tobacco mosaic virus in affected parts of plants has been determined in most cases by reinoculation on plants of *Nicotiana glutinosa* L. Virus content of infected plant tissues was determined when necessary by a previously described method (6, p. 47). This method utilizes the fact that the numbers of necrotic primary lesions developing on *N. glutinosa* leaves inoculated by rubbing with cloth saturated with tissue fluids indicate relative concentrations of virus.

The approximate location of virus in the tissues of some hosts has been studied by the use of iodine-staining of leaves of plants taken from greenhouse conditions at 5 p.m., stored in darkness at 10° C. until morning, and and then in darkness at 22° C. for 6 to 8 hours. This process, which has been described elsewhere in more detail (7, p. 166; 8, p. 297) shows the areas in which virus has recently begun to increase, because in such areas starch is retained for some hours longer than in tissues not containing virus.

CLASSIFICATION OF SYMPTOMS

The symptoms produced by plants in response to infection with the virus of tobacco mosaic may be classified as primary or secondary according to whether they occur at the site of inoculation or in some other part

of a plant. The symptoms thus far distinguished are described below under these two classes. With study of new host species, and more intensive examination of those already considered, additional types of response will no doubt be noted.

PRIMARY SYMPTOMS

Almost all infected plants show some symptoms at the site of infection. These primary symptoms may consist of necrotic lesions, yellowish lesions, lesions in which starch is retained abnormally long during storage in darkness, or lesions in which chlorophyll is retained abnormally long under conditions favorable to loss of this pigment from uninfected tissues; all of these lesions are similar in form, and apparently represent interference with normal functions in spots in which virus is increasing. Abscission of an inoculated leaf may also occur as a primary symptom of infection.

Necrotic lesions. In *N. glutinosa*, *N. acuminata* Hook., and a number of other species primary lesions occur as solid spots of necrotic tissue. In *N. glutinosa* the spots consist of a center of light brown and a dark brown peripheral zone. In *N. acuminata* the lesion is medium brown throughout. Occasionally necrotic primary lesions are in the form of necrotic rings of tissue with an enclosed area of green living tissue; this type of lesion occurs in *Physalis angulata* L., and at times in *N. rustica* L. and *N. langsdorffi* Schrank.

Yellowish lesions. Yellowish primary lesions may be more or less conspicuous on certain hosts according to environmental conditions. They appear on *N. tabacum* L. var Turkish during summer months, but are often difficult to see during other parts of the year. Generally they are not sharply outlined, but hazy in contour. They may be seen usually on young leaves of *Capsicum frutescens* L. var. Pimiento, although on older leaves which are becoming yellowed with age, they are replaced by greenish lesions which will be described later.

Starch retention lesions. Primary lesions marked by retention of starch during storage of plants in darkness have been seen in *N. tabacum* var. Turkish, in *N. glauca* R. Grah., in *Capsicum frutescens* var. Ruby King, in *Nicandra physalodes* (L.) Pers., and preceding necrosis in *N. rustica.* No other hosts have been examined carefully in this respect, but it is probable that many others may show such lesions. The technique of demonstrating starch retention has been described briefly above.

Chlorophyll retention lesions. Primary lesions marked by retention of chlorophyll have been found after inoculation on old yellowing leaves of *Nicandra physalodes* and of *Capsicum frutescens* var. Pimiento.

Abscission of inoculated leaf. In some varieties of *Capsicum frutescens*, such as Tabasco, in which necrotic primary lesions occur, the inoculated leaf is commonly lost a few days after infection, although uninoculated leaves of about the same age are retained. Sometimes before abscission

the leaf is turned down so as to point directly toward the base of the plant as a result of a backward bend in the petiole. *Physalis angulata* also shows the symptom of abscission of the inoculated leaf. Generally virus passes from the inoculated leaf before abscission occurs, but occasionally it is either lost with the inoculated leaf, or for some other reason is localized so that a plant grows to maturity without showing systemic symptoms.

Hosts in which no primary lesions are known. There are a few hosts in which no primary lesions are known. Some of these belong to the group of symptomless carriers. A typical example is *Solanum melongena* L. var. Hangchow Long in which no symptoms have been observed throughout the life of the plant, although increase of virus occurs in the inoculated leaves as well as elsewhere. Other instances of hosts in which no primary lesions are known belong in the group which show mottling; the garden tomato, *Lycopersicon esculentum* Mill., is an example of this kind.

SECONDARY OR SYSTEMIC SYMPTOMS

In addition to primary symptoms most hosts of the virus show systemic symptoms. The commonest of these systemic symptoms are mottling, leaf distortion, stunting of plants, necrotic secondary lesions, scattered yellowish spots, and lesions invisible in the living plant but demonstrable by staining with iodine after holding plants in darkness. Less common systemic symptoms are abnormal tissue outgrowths, defoliation, loss of flowers and fruit, prolonged yellowing, bending of stems, spotting of flowers, and failure to remove chlorophyll from secondary lesions.

Mottling, distortion, and stunting. Mottling symptoms are well-known from their occurrence in the hosts in which the virus was first recognized. They occur not only in tobacco and tomato, which have been much studied, but in many less-studied plants such as *Hyoscyamus niger* L., *Nicotiana tomentosa* Ruiz. & Pav., and *Solanum nigrum* L.

Leaf distortion is pronounced in some plants, such as *Nicotiana tabacum*, producing filiform leaves, large blisters of green tissue, and shrunken yellow areas. In other plants, such as *Solanum nigrum*, leaf distortion is inconspicuous or absent.

Stunting is severe on plants showing prolonged yellowing before mottling, such as *N. quadrivalvis* Pursh., and on plants showing conspicuous mottling with leaf distortion, such as *Hyoscyamus niger* and *N. tabacum;* it is inconspicuous or absent on symptomless carriers such as *Solanum melongena* var. Hangchow Long. Stunting is severe on plants showing numerous lesions of systemic necrosis, as in young plants of *N. rustica;* slight in plants showing few lesions of systemic necrosis, such as old plants of *N. rustica.* It is practically absent in plants such as *N. glutinosa,* which show localized necrosis, unless the necrosis destroys large areas of tissue and thus interferes with the food supply of the plant.

Necrotic lesions. Secondary necrotic lesions occur on such hosts as *N. rustica*, *Physalis angulata*, and young plants of some varieties of *Solanum melongena*. They are similar to the primary necrotic lesions on the same plants, except that they may fuse to form necrotic bands along veins and to form large dead areas.

Yellowish lesions. Yellowish spots, usually with vaguely outlined margins, are formed on many hosts on leaves too old to respond by mottling at the time of systemic spread of the virus. In *Nicandra physalodes* the virus seems to enter or multiply in the leaves only at this stage, so that a continuous succession of spotted leaves occurs. In other plants, as in *N. paniculata* L., *N. tomentosa*, and frequently temporarily in the beginning of systemic infection in *N. tabacum*, these yellowish spots are isolated and may become large without fusing. Commonly in *N. tabacum* the spots are so close together that they can be distinguished only on the edges of invaded areas, in the interior of which they appear only as bands along veins because of early fusion or of occurrence of centers of secondary infection so numerous as to be indistinguishable. These bands of tissue along veins are recognized as clearing of veins. In the course of a few days after the appearance of such bands along veins, the affected areas broaden, and the whole part of the leaf which was involved in clearing of veins becomes more or less uniformly yellowed. The tissue may later regain some of its original greenness.

Prolonged yellowing. Prolonged yellowing of young and middle-aged leaves is found in *Nicotiana quadrivalvis*, *N. clevelandii* A. Gray, *Capsicum frutescens* var. Ruby King, and other pepper varieties. It is associated with severe stunting and with delayed appearance of mottled leaves.

Secondary lesions invisible in the living plant, but demonstrable by staining. In many plants, as for example *N. tabacum*, the entrance of virus into uninoculated leaves which were old when the plant was inoculated is not followed by any easily distinguished symptoms in the living leaf, but starch retention along the edge of the invaded area may be demonstrated by storing plants in the dark and subsequently staining leaves with iodine.

Outgrowths of tissue. Abnormal outgrowths of tissue from the underside of mottled leaves occur in *N. tomentosa* and *N. paniculata*. The outgrowths often surround yellow areas of poor development in the mottled leaves.

Defoliation. Severe defoliation of plants is found in *Physalis peruviana* L. In this plant young and middle-aged leaves fall, but inoculated leaves and all other old leaves are retained. Defoliation also occurs in *Capsicum frutescens* var. Pimiento and other peppers, especially in plants infected in late summer.

Flower and fruit drop. Blossoms and young fruits are sometimes dropped by *Capsicum frutescens* varieties soon after systemic infection of plants. Blossoms and blossom buds are dropped by *Nicotiana quadrivalvis* for a

time after infection. In this plant distorted blossoms later develop, and set seed.

Hosts in which no secondary lesions are known. A few hosts are not known to show any symptoms of systemic infection. These fall into two classes. The first includes those which show primary necrosis only, and do not allow virus to spread from the immediate vicinity of the dead spots produced at the site of infection. *Nicotiana glutinosa*, *Datura stramonium* L., and old plants of *Solanum melongena* var. Black Beauty are typical examples of this. The second class includes hosts which allow virus to spread from the inoculated leaf, to locate in other parts of the plant and to multiply there, but which do not respond to the presence of the virus in these secondary centers of multiplication by any change from the normal condition. *Solanum melongena* var. Hangchow Long is a typical example of this symptomless carrier condition. Some plants of *Physalis alkekengi* L. appear also to be of this nature, but others show mottling symptoms on developing leaves.

SYMPTOMS NOT PREVIOUSLY DESCRIBED

Symptoms of infection with tobacco mosaic virus not previously described are prolonged yellowing, occurring in *N. quadrivalvis*, *N. clevelandii*, and some varieties of *Capsicum frutescens*; leaf abscission, occurring in *Physalis peruviana* and some varieties of *Capsicum frutescens*; flower and fruit drop, occurring in *Capsicum frutescens* varieties; outgrowth of tissue on the lower side of mottled leaves, occurring in *Nicotiana tomentosa* and *N. paniculata*; death of plants by systemic necrosis, occurring in young plants of *N. rustica* and of *Solanum melongena* var. Black Beauty; failure to remove chlorophyll from yellowing old leaves, occurring in *Nicandra physalodes* and *Capsicum frutescens* var. Pimiento; bending of upper stem toward side of plant bearing inoculated leaf, in *N. rustica*; and intensification of pigment in spots on flowers of *Nicandra physalodes*.

SYMPTOMS ON SPECIFIC HOSTS

The above described primary and secondary symptoms are found in host plants in such varied combinations that no adequate idea of sequence of symptoms can be given by discussion of symptom types. It is therefore necessary to recount for each of a number of hosts the order of appearance of symptoms, and to refer briefly to other hosts in which the symptom complex may be similar except in a few details. This is done in the following sections of this paper. It is hoped that the descriptions may be of use in distinguishing tobacco mosaic virus from other viruses, since the symptoms are described in greater detail than has been done previously for some of the less known hosts.

No claim can be made for completeness, since changes of environment

modify symptoms so that additional descriptions would be necessary if each infected species had been observed under a variety of growing conditions. Only in a few cases, as in *N. glutinosa* and *N. tabacum*, has this been possible, and even in these cases exposure of the plants to still other environmental conditions will doubtless result in the production of new complexes of symptoms. In most cases it was necessary to confine attention to the plants as grown under ordinary greenhouse and garden conditions.

The combination of symptoms in different hosts are so varied as to make classification difficult, but to facilitate reference, the various symptom complexes will be discussed under the following symptom groups: localized necrosis, systemic necrosis, masked symptoms, mottling, and yellowing. The symptom groups are nearly but not altogether mutually exclusive. Slight changes, as increase in age of plant, may cause the appearance of localized necrosis in hosts which ordinarily respond with systemic necrosis; this occurs in *N. rustica*. Mottling may appear in plants ordinarily of the masked symptom type, as in occasional specimens of *Physalis alkekengi*. Plants displaying mottling almost always show a trace of yellowing before mottling; this stage of preliminary yellowing may be seen in *N. tabacum*. All plants displaying yellowing in its most pronounced form, if they survive, eventually show mottling, although as a minor part of the symptom complex.

LOCALIZED NECROSIS

Nicotiana glutinosa. The symptoms of tobacco mosaic on *N. glutinosa* consist of necrotic primary lesions which have been described (6) in an earlier paper. At the point of introduction of the virus of tobacco mosaic into the leaf tissue of this plant a primary lesion develops in the form of a necrotic spot, first appearing as a tiny dark sunken area, which upon drying becomes light tan in color. On the fourth day after inoculation, at temperatures between 20° and 25° C., a ring of dark brown pigment develops on the periphery of the lesion. This ring makes the dead spot conspicuous and allows it to be distinguished from other lesions nearby even when so many are present that they fuse.

The amount of virus which can be recovered from these primary lesions is less than is produced at the site of infection in leaves of most host species. The localization of virus in the primary lesion of *N. glutinosa* is so effective that at greenhouse temperatures of 20° to 25° C. it has not proved possible to demonstrate the presence of virus in the green leaf tissues except in those immediately adjacent to lesions. This circumstance permits the isolation of single lesion strains of virus. In young plants during midsummer the primary lesion occasionally extends to the petiole of the inoculated leaf and the portion of the stem near the juncture with this petiole. It should be noted that in such cases it is still the primary lesion which is

observed, for the necrotic area simply elongates along vein, petiole, and stem. The systemic necrotic symptoms characteristic of *N. rustica* and some other hosts are different in that they have no visible necrotic connection between them and the primary necrotic lesion. Samuel (13) found that systemic spread of virus could be induced in this plant by raising the temperature to 35° C.

The temperature of the environment has been found to affect the development of primary lesions. At high temperatures, such as 30° C., the lesions are late in appearing, large and light colored. In unlighted incuba-

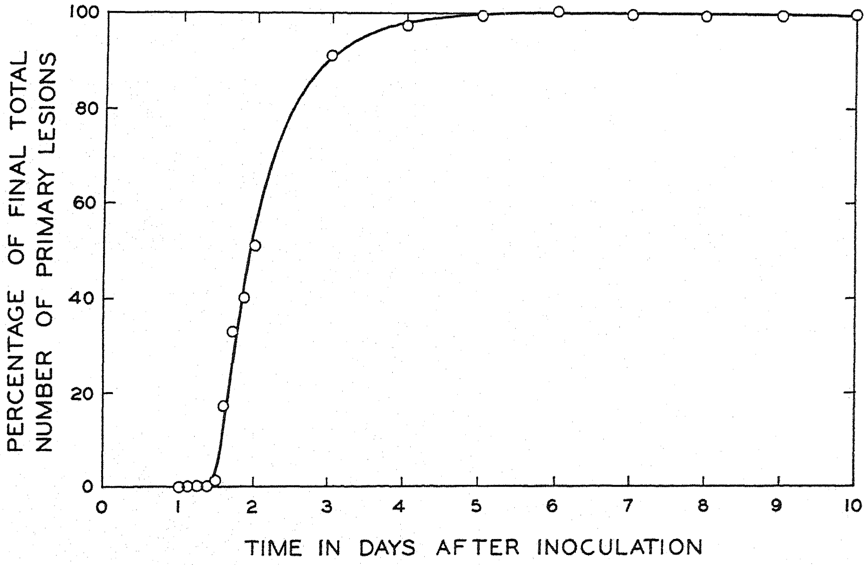

FIGURE 1. Time of appearance of 615 necrotic primary lesions on *Nicotiana glutinosa* leaves after inoculation with tobacco mosaic virus. Temperature approximately 24° C.

tors with which such temperatures have been maintained, it has sometimes been observed that the green leaves tend to become yellowed where the tissues are not invaded by the virus, but that certain spots remain green. These spots proved to be the infected areas or primary lesions. They may remain without necrosis for some time, but break down quickly when the plant is removed to ordinary room temperature. At slightly lower temperatures, such as 20° to 25° C., the spots are approximately of the type described as typical of this host. At still lower temperatures, such as 16° to 18° C., the spots are distinctly smaller and darker brown without the differentiation of light tan center and dark brown periphery, and they appear late. No spots develop at all at temperatures as low as 10° C.

Primary lesions on *N. glutinosa* appear very soon after inoculation,

slightly preceding the appearance of similar necrotic local lesions on other hosts of this virus. At the most favorable temperature, which seems to be approximately 23° C., the first lesions appear 30 to 36 hours after inoculation. The time of appearance of lesions at this temperature is represented in Figure 1. About half the number of lesions are visible at the end of 48 hours, and practically all are in evidence at the end of three days. A convenient rule for judging whether the lesions have all appeared is that all may be assumed to be visible when the outer ring of dark brown pigment begins to form on some of the lesions.

The physiological state of the inoculated leaf seems to have an effect on the appearance of the necrotic lesions. Leaves near the tips of branches, that is, young leaves, show the most striking lesions, with great contrast of color between tan-colored center and dark brown periphery. Older leaves show somewhat smaller lesions with less dark pigment at the periphery.

Other Nicotiana species. Nicotiana alata Link & Otto, *N. sanderae* Sander, *N. acuminata*, and *N. langsdorffi* frequently allow primary lesions, photographs of which have been published previously (6, p. 42; 7, p. 169) to extend to veins, petiole, and stem. Stem lesions sometimes cause plants to fall and later wilt on account of the damage to the stem tissues. In Figure 2 A to D is shown a photograph of four plants of *N. acuminata* inoculated in hot weather of summer; the plants display varying amounts of injury from stem necrosis.

Datura stramonium. In *Datura stramonium* localized necrotic lesions result from the inoculation of tobacco mosaic virus. These lesions resemble those described for *N. glutinosa*, being light tan in the center and dark brown on the periphery when they reach their best state of development. No symptoms of systemic nature appear on this host under the conditions to which it has been exposed. This species is commonly infected in the field by a mosaic disease which causes a conspicuous mottling and distortion of the leaves; this is not tobacco mosaic, as shown by its failure to produce symptoms typical of the disease on transfer to *N. glutinosa*. A picture of tobacco mosaic lesions on *D. stramonium* is included in an earlier paper (7, p. 169, Fig. 3 N). The necrotic lesions on *D. stramonium* differ in their late appearance after inoculation from those on *N. glutinosa*, which they resemble in appearance. The lesions generally appear about a week after inoculation. This comparatively late appearance of the necrotic spots greatly reduces the usefulness of this plant for laboratory tests, for which it is otherwise well suited, because it is easily grown from seed and shows conspicuously pigmented lesions.

A response to infection similar to that shown by *Datura stramonium* is given by *Solanum pseudo-capsicum* L. A photograph of the necrotic primary lesions is represented in an earlier paper (7, p. 169, Fig. 3 K).

Solanum melongena var. Black Beauty. Some varieties of *Solanum*

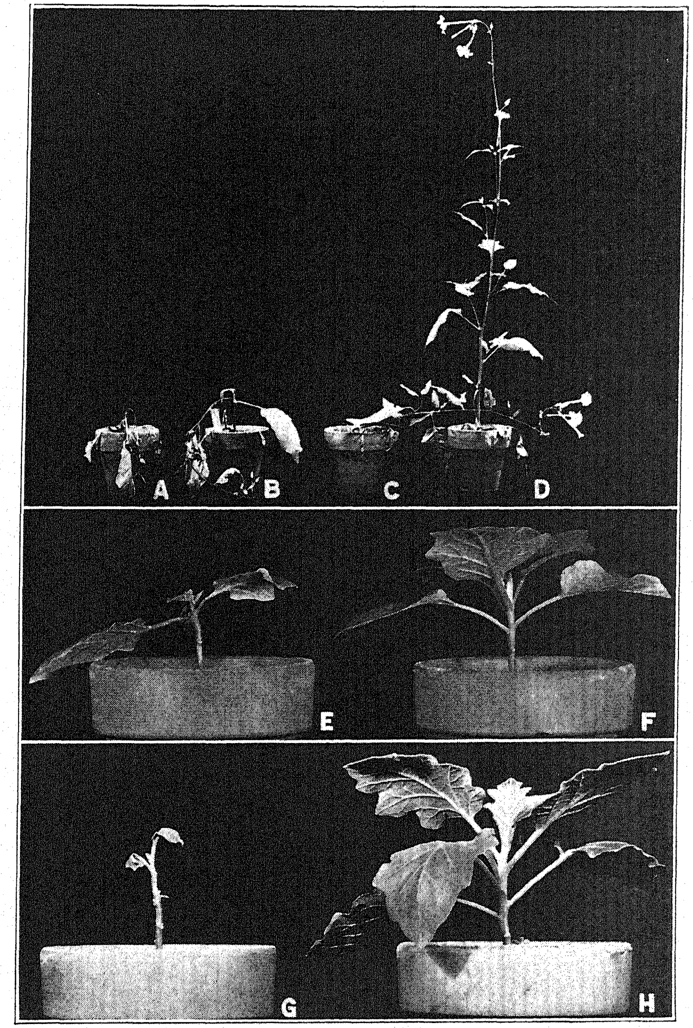

FIGURE 2. A to D: Four plants of *Nicotiana acuminata*, showing injury from necrotic lesions in stem. A, Stem extensively involved by necrosis, leaves dying; B, Necrosis on stem above and below petiole of inoculated leaf, stem fallen, leaves wilted; C, Like B, but leaves not wilted; D, Necrosis on stem below petiole of inoculated leaf, stem erect, leaves and flowers normal; E to H: Plants of *Solanum melongena*, showing systemic necrosis. E, Plant 4 weeks after infection, necrosis on stem and necrotic spots on leaves; F, Uninoculated control plant of same age; G, Same plant as E, but 7 weeks after infection, largely defoliated, and much marked with necrosis. This plant died 8 weeks after infection; H, Control plant at age of G.

melongena produce localized necrotic lesions after inoculation with tobacco mosaic virus. Certain other varieties of the species respond differently; the varieties Peking Green and Long White show obscure mottling; the varieties *creviviolacea*, Kuli, and Hangchow Long are symptomless carriers.

Young plants of *Solanum melongena* var. Black Beauty are much more severely affected by inoculation with tobacco mosaic virus than are older plants. This is because virus moves through such plants to produce a systemic necrosis, but is completely localized in the leaves of old plants. In other species in general it has been found that movement of virus from infected leaves to other parts of plants occurs consistently later in old plants than in young. In this species, however, movement of virus in young plants often results in death of the entire plant from systemic necrosis, which involves leaves near the top of the plant and portions of the stem. Localization of virus in the slightly older plant is so complete that inoculation does little harm, because loss of the small amount of tissue involved in the primary lesions does not noticeably affect development of the remainder of the plant.

Necrotic lesions on old plants of *S. melongena* var. Black Beauty appear three or more days after inoculation. They are dark brown in color and rarely become larger than four millimeters in diameter. Localization is complete on leaves of plants more than four or five inches high at the time of inoculation. Even the youngest leaves of old plants are capable of localizing virus with which they are inoculated, and so differ from the somewhat similar leaves of younger plants. A photograph of the necrotic primary lesions on a leaf of this plant has been shown in a previous paper (7, p. 169, Fig. 3 L).

Phaseolus vulgaris L. Price (12) has shown that on many varieties of the garden bean, *Phaseolus vulgaris*, inoculation with tobacco mosaic virus produces strictly localized necrosis with the formation of a reddish pigment, and that certain other varieties do not respond to inoculation with this virus.

The striking features of the localized necrosis in the bean varieties are production of a reddish pigment, very small size of all lesions, comparatively low virus content of infected leaves, and absence of extension of lesions to involve large veins, petioles, or stem portions. Price has shown that the primary lesions may be utilized to estimate virus concentration, and that the plants are so easily grown as to have an advantage over *N. glutinosa* in this respect.

The varieties found by Price to be susceptible to tobacco mosaic may be separated into three groups, the first of which forms reddish local lesions a little larger than others, although the lesions rarely exceed a millimeter in diameter. This group includes Early Golden Cluster, Scotia, Refugee Green Pod, and Boston Pea. The second group, which regularly forms very

tiny reddish primary necrotic lesions, includes Ideal Market, Cut Short, White Creaseback, Stringless Refugee, Hobson Long Pod, Keeney's Stringless Refugee, and New Navy Robust. The third group sometimes fails to produce lesions on individual plants, although other individuals are susceptible; it includes Unrivaled, Improved Round Pod Valentine, Great Northern, Refugee Extra Early, and Full Measure.

SYSTEMIC NECROSIS

Solanum melongena var. Black Beauty. Young plants of *Solanum melongena* var. Black Beauty are often killed by the systemic spread of tobacco mosaic virus following its introduction into leaf tissue. Primary necrotic spots occur in the inoculated leaves of young plants as in those of old specimens, and the lesions are similar in appearance in the two cases. In young plants of this variety necrotic spots and necrotic streaks along veins appear in developing leaves some days after the appearance of primary lesions on the inoculated leaf, and portions of stem near the top of the plant are often involved. Some defoliation may take place, leaves with secondary lesions dropping off and leaving the stem with only the smallest leaves at the top. Stem lesions often occur near the petiole of the infected leaf. In such cases the lesions extend further along the stem below the petiole of the infected leaf than above it. They are narrow strips of dead tissue, generally narrower than the petiole itself, tapering off to rounded ends. When plants have been affected by the systemic disease for some time, stem lesions and dead streaks in petioles and midveins may be numerous and conspicuous. In cross section the dead tissues are seen to be superficial in some cases and in deeper tissues in others.

Plants are generally killed if inoculated when but two or three leaves are well formed in specimens approximately two inches high. Death of young plants by systemic necrosis has not been commonly recognized as a symptom of infection by tobacco mosaic virus. Specimens three inches high may develop the systemic necrosis and be killed finally but the process is less uniform and some plants remain alive much longer than others. The critical point is reached when the plants are approximately four inches high; at this stage in the greenhouse at approximately 22° C. the plants have reached a size at which they may be infected without the development of the systemic form of the disease. Large plants have been inoculated heavily even on their youngest leaves, and subsequently grown to maturity but systemic symptoms have never been observed in them. The increase of resistance to systemic infection with age is conspicuous in this plant.

In Figure 2 E to H photographs illustrating two stages in the development of the systemic disease in a young plant are shown.

Nicotiana rustica. In *Nicotiana rustica*[1] systemic necrosis in young plants generally leads to the death of the top of the stem with the developing leaves, and kills the plant. In half-grown plants upper leaves are invaded by the virus, which causes the formation of necrotic spots and interferes more or less with growth, but with increase in age of the plant it is common to have an unharmed flower shoot develop and produce seeds. In very old and pot-bound plants inoculation may not produce systemic disease. Only much hardened and pot-bound plants, however, seem to be capable of localizing the infection completely. Large, succulent, field-grown specimens may be killed by development of extensive patches of necrosis in the upper stems and growing leaves. In this respect the species does not act at all like *S. melongena* var. Black Beauty in which no degree of succulence in the old plant has ever been observed to allow the development of systemic necrosis.

In young plants of *N. rustica* from two to five days after infection, starch retention lesions can be demonstrated in inoculated leaves. About the fifth day after inoculation necrotic primary lesions appear, followed soon by a phenomenon of clearing of veins somewhat similar to that known in *N. tabacum*. The youngest leaves are retarded in their development, begin to pucker, and appear abnormally yellowish in color. The puckering appears to be due to the somewhat greater detrimental effect of virus on growth of veins of young leaves than on growth of intervenal tissue. Veins of developing leaves are often affected by necrosis. The youngest leaves die soon after virus reaches them. In general the attempt to put out new leaves is followed by recurring necrosis of so severe a sort as to culminate eventually in the death of the plant. In some cases buds at the base of the stem continue to develop green leaves for many days, but these also are finally invaded. In general plants which are infected while young do not succeed in throwing off the disease and growing to maturity.

In Figure 3 A to J are shown plants of *N. rustica* of several ages affected by systemic necrosis in comparison with healthy plants of the same ages. In *N. rustica* there is frequently bending of the top of the plant toward the inoculated leaf, a symptom similar to that called "crookneck" by Guterman (5) in his description of mosaic on *Lilium auratum* Lindl. Necrosis at the top of a large but immature and rapidly growing plant of *N. rustica* is frequently restricted to leaves of the sector immediately above the inoculated leaf. About a day before the appearance of necrotic lesions on the young leaves at the top of the plant, growth of the succulent portion

[1] It must be pointed out that the species *N. rustica* has many varieties, and that among these there is probably some variation in response to the virus of tobacco mosaic. The variety used by the writer is an unnamed one, characterized by rapid growth and large size. Seed of this strain will be furnished upon request to anyone who may wish to study it.

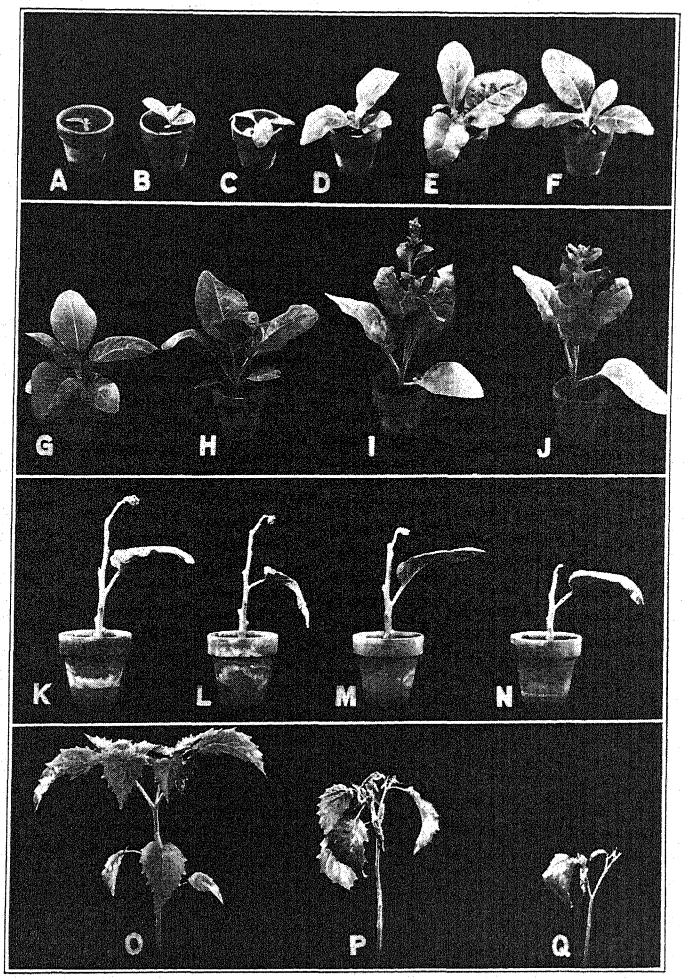

FIGURE 3. A to J: Plants of *Nicotiana rustica* of various ages, showing effects of systemic necrosis. A, Young plant, nearly dead and little larger than when inoculated; B, Uninoculated control of same age; C, Plant two weeks older when inoculated than those in A and B, center leaves of plant dead; D, Uninoculated control of same age as C; E and F, G and H, I and J, similar pairs of inoculated and uninoculated plants, each two weeks older than the preceding pair, showing less harmful effect of inoculation of older plants. In I only a few necrotic spots represent the systemic disease; K to N, Four plants of *N. rustica*, all leaves, except the one inoculated, removed to show "crookneck" symptom consisting of bending of upper stem in the direction of the inoculated leaf; O to Q: Defoliation accompanying systemic necrosis in *Physalis angulata*. O, Uninoculated control plant; P, Inoculated plant which has lost some leaves; Q, Similar plant almost completely defoliated.

of stem near the top is retarded on the affected side so as to cause bending, which progresses until the tip of the stem may actually point downward. The stem, when first bent, does not show internal macroscopic necrotic lesions, although in time the upper portions of the stem as well as the leaves at the top of the plant become involved by severe systemic necrosis. In Figure 3 K to N are photographs of four plants of *N. rustica*, with all leaves except the inoculated one on each plant removed to show the stem as clearly as possible. It will be seen that bending of the stem toward the inoculated leaf had taken place in each plant. Necrotic lesions were present on young leaves on the same side of the stem at the top and on young leaves of axillary shoots on the same side of the stem.

Physalis angulata. In the genus *Physalis* are found species showing many types of response to infection with tobacco mosaic virus. One species, *Physalis angulata*, shows systemic necrosis somewhat similar to that shown by *Nicotiana rustica*. *Physalis angulata* develops small necrotic primary lesions on its inoculated leaves about three days after inoculation. The lesions break down more completely on the older leaves, appearing imperfectly as traces of necrosis on younger leaves. The lesions are sometimes solid necrotic spots, and sometimes delicate rings or double concentric necrotic rings with green tissue between the rings of necrosis.

Defoliation follows the appearance of these primary lesions, all inoculated leaves being lost within a week or two of the time of inoculation. Loss of the inoculated leaf does not occur in *N. rustica*, and does not occur often in *Solanum melongena* var. Black Beauty. This loss of the inoculated leaf in *P. angulata* does not prevent the spread of the virus, at least in most instances. Many of the inoculated plants soon show necrotic lesions on the basal portions of younger leaves, with resulting dropping of these leaves, only the oldest and the very youngest leaves being temporarily unaffected. In time the older leaves are lost. The stems then stand for from one to three weeks, bearing tiny leaves near the growing points which do not enlarge, but finally die. Later nearly all affected plants die, generally collapsing first at a point just above the surface of the ground. After shedding the inoculated leaf a few plants remain for a greater or less time without secondary symptoms. In some of these cases a little of the virus must pass the petiole before abscission, for systemic necrosis appears after a short delay, and the plant dies as above described. In a few cases the virus is either lost by abscission of the inoculated leaf, or so localized as to be harmless until the plants are able to blossom and mature seed. In a few cases also this plant illustrates the close relationship between systemic necrosis and mottling by showing a sort of mottling pattern flecked with necrosis on young leaves developing after defoliation of stem. In Figure 3 O to Q are represented the tops of a healthy *Physalis angulata* plant and of two infected plants of the same species, one showing necrosis

of middle-aged leaves from the systemic infection, and the other almost complete defoliation.

Other hosts showing systemic necrosis. Certain varieties of potato, *Solanum tuberosum*, such as the variety Green Mountain, are affected by systemic necrosis as a result of inoculation with tobacco mosaic virus. The infection somewhat resembles that on young plants of *Nicotiana rustica*. A small amount of systemic necrosis occurs on certain varieties of the garden pepper, *Capsicum frutescens*. Thus the varieties Anaheim Chili, Tabasco, Creole, and a pepper grown under the name of *Capsicum minimum* Blanco have shown some systemic necrosis, although there is a considerable tendency for the necrosis to be confined to the primary lesions in some of these varieties, notably Tabasco pepper. The four pepper varieties named above show an abnormal position of the inoculated leaf a few days after infection. The petiole bends backward so that the leaf, instead of pointing outward and slightly upward, points straight down. Abscission generally occurs shortly after this position is assumed.

MASKED SYMPTOMS

The first description of a completely masked or symptomless infection of any species of plant with tobacco mosaic virus was that of the infection in *Physalis alkekengi* reported by Nishimura (11) in 1918. He showed that inoculated plants, although healthy in appearance, allowed virus to multiply in them to such an extent that they were good sources of virus for infecting other plants. Previously Allard (2) had reported that *Nicotiana glauca* produced mottling when first infected as a young seedling, but later became symptomless though still infectious. Among the plants recently studied there have been a number of species which may be classed as symptomless carriers. These plants are not necessarily always without detectable symptoms, as will be illustrated in the case of species to be described in this section, but they are all without visible external symptoms at times.

It was not possible for the earliest studies to include accurate estimates of the concentration of virus developed in symptomless hosts. It has been found, however, as pointed out in the following discussions, that symptomless plants are sometimes capable of developing high concentrations of virus, especially in inoculated leaves. A study of virus distribution, as well as examination of patterns formed by starch retention during brief storage in darkness, has suggested an explanation of the symptomless character of the infection in these plants. In most cases symptomless hosts of tobacco mosaic virus seem to allow the virus to spread toward the top of the plant with difficulty, so that it never enters, or at least never comes to great concentration in, very young growing tissues. There seems to be a tendency for virus to spread in small amounts and erratically to leaves of medium

size considerably below the growing points at the top of the plant. In these leaves virus perhaps develops to high concentration only after the leaf has become too old to be modified in shape. Symptomless hosts have the further characteristic of not showing, or not always showing, yellow spots when virus comes to considerable concentration in old leaves. These two characteristics, absence of high concentration of virus in very young tissues and failure to yellow at the site of virus increase in old leaves, seem to be commonly associated with, and possibly responsible for, the symptomless carrier condition.

Solanum melongena var. Hangchow Long. An excellent example of the symptomless carrier condition was found in the Hangchow Long eggplant. Inoculation of a leaf of this plant resulted in the early production of a large amount of virus locally without affecting the appearance of the inoculated leaf, and promptly initiated secondary infection in some other leaves. Plants inoculated as very young seedlings were kept under observation until fruit had ripened. No symptoms were seen at any time.

The concentration of virus was determined in various portions of a plant five weeks after inoculation. Each portion was tested by inoculation of five leaves of *N. glutinosa* with extracted juice. The results of these tests are given in Table I. As may be seen from this table, the youngest leaves contained small traces of virus, partly expanded leaves contained moderate amounts of virus, and large leaves, except those uninoculated but already fully expanded at the time of inoculation, contained much virus. Leaves uninoculated but already fully expanded at the time of inoculation showed small amounts of virus. The greatest accumulation of virus appeared in the inoculated leaf. The roots and stems were found to contain considerable amounts of virus. It was found that the stem contained little virus near its tip, more some distance below the tip, and much in the lower parts. Measurements of the same kind on additional plants gave similar results.

This tendency of the virus to appear in quantity in well developed leaves, but not to be present in large amounts in younger leaves, constitutes part of the evidence for the belief that in symptomless carriers virus does not reach, or does not increase concentration in, the dividing cells at the growing point, but invades new leaves after they are mature enough so that distortion and mottling do not occur. The tendency of the stem portions to show a similar gradient of virus suggests the possibility that the stem shares the mechanism, whatever it may be, which prevents virus from reaching, or from multiplying in, very young tissues in this host.

A similar symptomless condition was found in *S. melongena* var. *creviviolacea* and in *S. melongena* var. Kuli infected with tobacco mosaic virus, and a similar gradient of virus was demonstrated in successive leaves of these plants.

TABLE I

TOBACCO MOSAIC VIRUS IN SYMPTOMLESS EGGPLANT (SOLANUM MELONGENA VAR. HANGCHOW LONG)

Portion of eggplant tested	Number of lesions produced on *N. glutinosa* by inoculation with juices from:									
	Tip leaves and young stem	First leaf below tip; internode below leaf	Second leaf; internode below	Third leaf; internode below	Fourth leaf; internode below	Fifth leaf; internode below	Sixth leaf; internode below	Seventh leaf; internode below	Eighth leaf; internode below	Roots
Leaf	0	20	127	583	546	869	802	1393*	691	—
Internode	0	1	36	477	407	650	587	760	246	—
Root	—	—	—	—	—	—	—	—	—	102

* Test of inoculated leaf.

TABLE II

COMPARISON OF VIRUS CONTENT OF INOCULATED AND TOP LEAVES OF OLD NICOTIANA GLAUCA PLANTS

	Number of lesions produced on *N. glutinosa* by inoculation with juices from samples tested											
Days after inoculation	1	2	3	4	5	7	10	14	21	28	35	42
Inoculated leaves	0	14	70	223	535	508	784	995	2400	2160	1431	1354
Top leaves	0	0	0	0	0	0	9	2	0	2	19	3

Physalis alkekengi. In *Physalis alkekengi* the inoculated leaf, although unchanged in color, soon after inoculation contains a very large amount of virus. This species has not been studied intensively by the writer, but in some plants in which virus increase in the inoculated leaves was detected by successive inoculations to *N. glutinosa*, no virus was demonstrated in the top of the plant nor in underground stolons up to the 14th day after infection. This observation suggests that the movement of virus from the inoculated leaf is not as early as from inoculated leaves of some hosts of the virus. Virus was later detected in upper leaves of inoculated plants not showing symptoms. It was found that virus was more concentrated in juices of uninoculated old leaves than in juices of uninoculated young leaves.

One lot of plants of *P. alkekengi*, which were symptomless for a time after infection, temporarily showed intense mottling, and later produced new leaves which were symptomless. *N. glutinosa* plants inoculated with extracts of the mottled leaves produced many necrotic lesions, but similar plants inoculated with extracts of the subsequently-formed symptomless leaves produced few necrotic lesions. Thus it appears that plants of the species *P. alkekengi* may have virus in their uninoculated leaves without showing symptoms, but that at times the plants may lose their symptomless character and become mottled. The species deserves much further study, for under some conditions it appears to be a typical symptomless host of the virus. The species was found difficult to grow under greenhouse conditions during part of the year because of its susceptibility to attack by mites and because of its tendency to store food in rhizomes instead of producing leaves and blossoms.

Nicotiana glauca. The reaction of *Nicotiana glauca* to infection with tobacco mosaic virus differs from that of *Physalis alkekengi* and of the above described eggplant varieties in that this host shows a faint but typical mottling pattern when inoculated as a young plant. As infected plants grow, however, the mottling becomes very inconspicuous and in time may disappear from many of the plants. Plants still showing mottling after a period of growth always show virus in their mottled leaves. Those showing no further mottling may or may not contain virus in their upper leaves. The occasional failure of virus to be present continuously in the upper leaves of formerly mottled plants seems to indicate that in this host the virus is sometimes unable to keep up with the growth of the host plant. Further evidence of some factor for difficulty of movement of the virus within the tissues of *N. glauca* is given by results of measurements of virus in inoculated leaves and tip leaves of half grown plants. A series of measurements of virus content of inoculated leaves and top leaves of inoculated plants is given in Table II. On each day the inoculated leaf of a plant was tested, and a short piece of stem from the top of the same plant was tested

also. This tip of the stem carried with it several small leaves, none of them more than 1 inches in length. The increase of virus in the inoculated leaves was regular and rapid. The upper stem and young leaves showed no evidence of containing virus during the first week after inoculation of the lower leaf, and no evidence of containing much virus during the first six weeks.

Secondary lesions in *N. glauca*, as demonstrated by staining starch retention patterns with iodine, are frequently separate spots. They are at times rather numerous except on the very youngest leaves. On some individual leaves, and on successive leaves of plants, the lesions show a size gradient. In the younger tissue near the base of each leaf the lesions are small as compared with those near the apex of the same leaf. On younger leaves the lesions are small as compared with those on older leaves. On the youngest leaves very few lesions can be seen and those are very small. Possibly this gradient is caused by some opposition to movement of virus into intervenal leaf tissues when they are very young. Virus may pass occasionally into leaf tissue after it has reached a relatively advanced state of development, forming so few secondary lesions that these remain well separated for some days after their formation. If it may be assumed that the secondary lesions develop in diameter about as fast as primary lesions would on such leaves, it seems reasonable to believe that the gradient of sizes of lesions on individual leaves and on successive leaves is due to the different periods of time since the leaves were of an age favorable for virus invasion. As in Hangchow Long eggplant, this freedom of young leaves from invasion by virus may account for the masked condition of the infection. In *N. tabacum*, in which developing leaves are mottled and distorted soon after the onset of the disease, very small leaves are known to be invaded since they often show mottling. Leaves of *N. glauca*, however, are perhaps not invaded so early. They give no evidence of containing large or numerous secondary lesions until they are already well expanded and are perhaps no longer susceptible to distortion and marked mottling.

Lycopersicon pimpinellifolium Mill. The red currant tomato, *Lycopersicon pimpinellifolium*, allows virus to increase in its tissues to a high concentration, although not to so high a concentration as is attained in tissues of the varieties of *L. esculentum*, the common garden tomato. In spite of the fairly high virus content, *L. pimpinellifolium* shows little harm from the infection. Infected and uninfected plants produce leaves and branches of the same size, shape, and position on the plant, show no distinct mottling except in very young infected plants, and bear equally numerous fruits which ripen simultaneously.

Soon after infection of young plants an obscure but typical mottling of young leaves is discernible. This mottling cannot be identified with

certainty after a short time. The study of the distribution of virus in this host has not been undertaken.

In Table III successive measurements of the virus content of extracts of whole plants of *L. pimpinellifolium* are compared with the average measurements of virus content of plants of 15 varieties of *L. esculentum*. *L. pimpinellifolium* produced less virus during the first 40 days after infection than any one of the varieties of *L. esculentum* included in this study. This

TABLE III

COMPARISON OF VIRUS CONTENT OF MOSAIC PLANTS OF LYCOPERSICON PIMPINELLIFOLIUM AND OF L. ESCULENTUM

Days after inoculation of *Lycopersicon* plants	Number of lesions produced on *N. glutinosa* by inoculation with juices from whole plants of:	
	L. pimpinellifolium	*L. esculentum**
2	0	1
4	0	1
6	0	8
8	2	19
10	0	316
12	35	323
15	12	642
21	16	371
28	71	366
35	328	1573
42	487	2108
56	2084	1730
70	505	1725
84	1598	2021

* Average for 15 varieties of *L. esculentum*.

slightly lower virus content appears to indicate some resistance to increase of virus. Although the tendency toward resistance to virus production shown by *L. pimpinellifolium* is not very great, it may be of some value, because means are now at hand to make quantitative measurements of virus content by study of a few leaves without destroying individual plants. This gives some hope of securing the tendency toward repression of virus production in hybrid plants of the two species.

To summarize the descriptions of masked symptom hosts, it may be stated that in *Physalis alkekengi* virus multiplies rapidly in inoculated leaves and is late in producing a systemic infection; in *N. glauca* secondary infection of the upper part of the plant occurs, but erratically; in several eggplant varieties secondary infections occur promptly, but the virus does not appear in great quantity in the youngest tissues of leaf or stem. No mottling occurs in the eggplant varieties mentioned; in *L. pimpinellifolium* obscure mottling occurs in young plants but fades in older ones; in *N. glauca* mottling occurs in the young seedling soon after infection, but disappears in some cases as the plant grows older; in *Physalis alkekengi* old

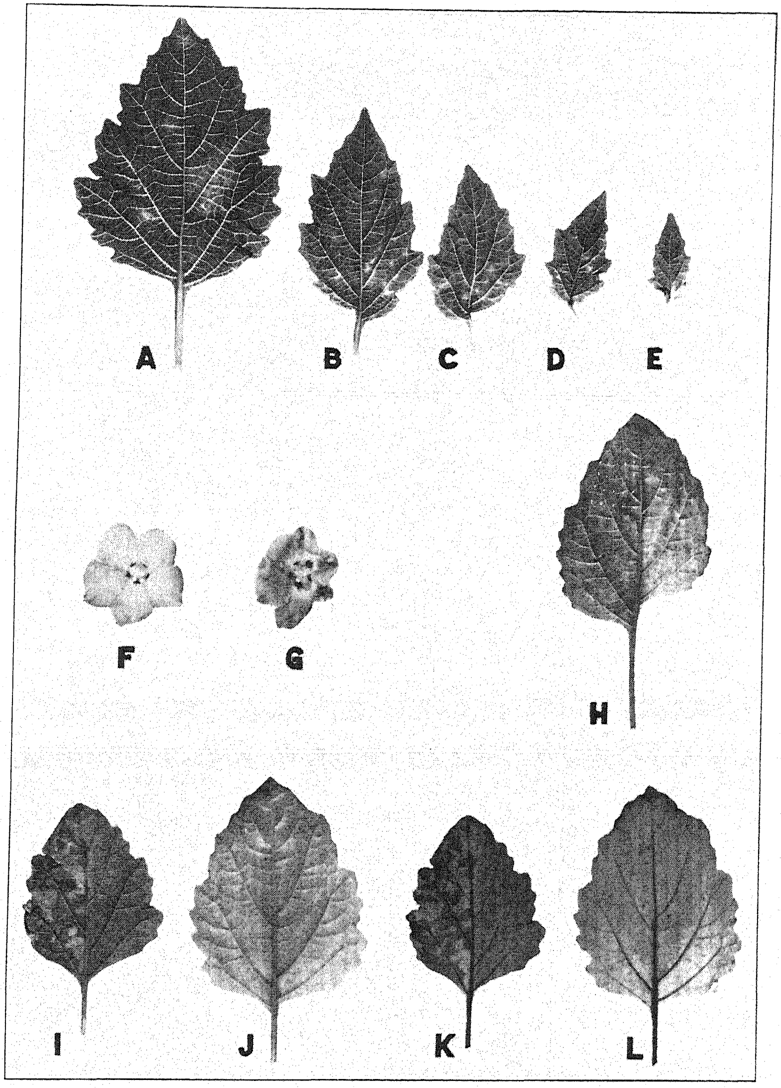

FIGURE 4. A to E, Leaves of a plant of *Nicandra physalodes* showing yellowish spots characteristic of systemic infection; F, Normal flower of *N. physalodes;* G, Flower with secondary lesions on distorted corolla. Lesions blue, normal corolla pale blue; H, Primary lesion extending as far toward apex as toward base of leaf, 18 days after inoculation by pin puncture. Lesion marked by chlorophyll retention in leaf which turned yellow with age; I, Leaf inoculated by rubbing during period of yellowing with age, showing retention of chlorophyll in primary lesions; J, Normal yellow leaf; K and L, Same leaves as in I and J, stained in iodine after being in sunlight; starch present only in the lesions.

plants may pass from a symptomless to a mottling response or vice versa. Masked carriers seem to be characterized by a tendency to prevent entrance or development of virus in the youngest tissues, thus avoiding distortion or mottling from the presence of virus.

Nicandra physalodes, not a symptomless host. Nicandra physalodes is similar to the symptomless carriers in allowing only erratic spread of a small amount of virus to the partly developed leaves near the top, producing few, discrete, secondary lesions on medium-sized and large leaves; but differs from them in allowing the chlorophyll content to be affected in these centers of secondary infection, with the production of a yellow spotting systemic disease (Fig. 4 A to E).

In this plant movement of virus is so poor that older plants inoculated heavily, or young plants inoculated lightly, may never show any systemic symptoms. To insure systemic symptoms, young plants not much more than an inch in height must be inoculated heavily. When only one or two primary lesions are produced on a leaf they frequently enlarge, as in plants of *N. tabacum*, and elongate when they involve veins, but the tissue involved along the vein toward the leaf periphery is frequently as extensive as that involved toward midvein or base of leaf (Fig. 4 H). It is believed that this type of elongation is part of the growth of the primary lesion itself, and dependent upon the shape of the cells concerned. It seems to be distinct from the condition of secondary spread which occasionally occurs in this plant, in which virus goes to distant leaves and flower parts, setting up discrete secondary lesions apparently not connected with the primary spots.

Secondary lesions occur on *Nicandra physalodes* not only on the leaves, where they appear as yellowing spots, but on blossoms, where they become visible by increase in intensity of the blue-violet color of the corolla (Fig. 4 F and G). Production of spots of deeper than normal color on flowers has not previously been recognized as a symptom of infection by tobacco mosaic virus.

Both primary and secondary lesions on the leaves of this host are remarkable, because, although they appear as yellowish lesions on young green leaves, they often show themselves as green patterns on old yellowing leaves which are losing chlorophyll with age. This production of green lesions on yellowing leaves, not previously reported as a symptom of tobacco mosaic on any host, appears to be due to interference with the normal rate of loss of chlorophyll in tissues containing virus. The patterns formed by chlorophyll retention are similar in contour to starch retention patterns in younger leaves, and like these indicate the approximate area occupied by virus. In Figure 4 I to L is represented a lower leaf of a plant with green lesions on a yellow background (I), in comparison with a similar but uninoculated entirely yellow leaf (J). The same two leaves are also

represented as they appeared after iodine staining to show starch distribution (K and L).

Plants of *N. physalodes* inoculated when past the seedling stage generally show few or no lesions of secondary or systemic infection. When such plants were defoliated except for the inoculated leaf and the small leaves near the growing point, however, more secondary yellowish lesions appeared and a few leaves showed mottling and distortion. Movement of virus to the top of the plant seemed thus to be aided by the removal of uninoculated expanded leaves. This suggests that after defoliation the inoculated leaf may have furnished a larger part of the food needed for the formation of new leaves at the top, and that the virus may have gone with the food or may have been aided by the conditions arising while the inoculated leaf was supplying such food.

The yellow spots characteristic of the infection in *Nicandra physalodes* are so located that they might be said to resemble the symptoms on *Physalis angulata* except that yellowing instead of necrosis occurs. The yellow spot infection on this host may be related to clearing of veins in mottling hosts. The secondary lesions in mottling hosts are usually so numerous along the veins of partly expanded leaves as to fuse into bands, and invasion of still younger tissues soon prevents the continued production of spotting symptoms. In mottling hosts, such as *Nicotiana tabacum*, however, well separated yellow spots are present frequently in the early stages of the systemic infection (7, p. 167, Fig. 2 G; 8, p. 315, Fig. 6 B, C, and I); and in *N. tomentosa* large, well separated yellow spots are characteristically present before mottling appears in the systemic infection. The yellow spotting would resemble the symptoms on *N. glauca* if it were not for the fact that intense yellowing occurs in all secondary lesions, whereas in *N. glauca* yellowing is faint and does not occur in all secondary lesions.

A similar yellow spotting infection occurs in *Lycium ferocissimum* Miers. In this plant also yellowish primary lesions appear on the inoculated leaf, and in the systemic infection yellow spots become visible on leaves just below the youngest, most rapidly expanding leaves. The affected tissues do not expand as rapidly as the green tissues in this plant, and the affected leaves roll their margins upward.

MOTTLING

Nicotiana tabacum. The symptoms of the common field type of tobacco mosaic on *Nicotiana tabacum* are perhaps as well known as the symptoms of any virus disease on any plant, having been studied for many years and described in connection with numerous studies of the virus.

Young and vigorously growing plants of *N. tabacum* show very faint yellowish primary lesions on inoculated leaves two or three days after inoculation. These lesions mark spots where virus has begun to increase

in concentration in the host. Iodine treatment has been found (7, 13) to disclose disturbances of starch distribution at these points.

Later, generally 3 to 7 days after inoculation, abnormally light color in tissue adjacent to veins, known as clearing of veins, appears in one or several of the young leaves of each plant unless sunlight intensity is very low. Plants in which virus has become systemically distributed during very cloudy weather show first a slight twisting of leaves less than one inch in length, then puckering of these leaves, and later the development of mottled leaves without clearing of veins. Clearing of veins is often confined to half of one or more of the growing leaves, or is much more pronounced on one side of a midvein than on the other; the half more affected is the half nearer to the side of the stem bearing the inoculated leaf. Cleared veins are involved in disturbance of starch removal (7, 13), a phenomenon which seems to characterize all areas of leaf previously healthy but recently invaded by virus. The abnormally light color of veins becomes much less conspicuous two or three days after its first appearance. Leaves affected by clearing of veins do not become distorted in outline; in color they are not as green as similar but uninfected leaves, and they remain yellowish-green after the cleared veins have become inconspicuous. Virus advances slowly toward the periphery of these leaves, faster along veins than between them, forming an oak-leaf pattern visible because of starch disturbances in stained leaves, and sometimes discernible in living leaves because the uninvaded peripheral area is greener than the invaded portion in the center of the leaf.

During the period of clearing of veins and for a few days after the clearing has become less conspicuous than at first, the growth rate of the plant is reduced, resulting in a stunting of infected plants which is evident upon comparing them with uninfected specimens of the same lots. This stunting is so severe as not to be entirely overcome in subsequent growth of the plant. During the latter part of this stunting period, mottled and distorted leaves begin to appear.

The leaves which mottle and show distortion need hardly be described, having been treated in detail by Goldstein (4). It may be mentioned, however, that in the mottled leaves relatively low virus content has been found in the green areas, and these areas are found to show a small number of discrete starch lesions when stained with iodine, apparently indicating the gradual leakage of small amounts of virus into areas temporarily capable of excluding secondary infection from nearby areas of high virus content.

After mottled and severely distorted leaves have been produced, a number of nearly green leaves are formed under conditions which have existed during some years of the writer's observation of *N. tabacum* var. Turkish. These grow to fair size before much yellowing appears on them.

The large green areas on these, like the large green blisters on the first distorted leaves, gradually show invasion by pale yellowish spots, and if stained in iodine show clearly defined spots of marked starch retention. There is therefore some reason to believe that immediately following and perhaps because of the formation of a few severely mottled and distorted leaves there is a short period during which a number of leaves are produced under conditions not favorable to invasion by virus.

This stage of production of nearly green leaves is transient, and is followed by a period in which moderately mottled and slightly distorted leaves are produced. This period is of indefinite extent, being terminated only by the death of the host plant from other causes.

The phases distinguishable in observed infections on *N. tabacum* var. Turkish are therefore five: first, formation of primary and temporarily localized lesions; second, clearing of veins and blanching; third, mottling and severe distortion; fourth, production of more nearly normal green leaves, which subsequently acquire yellowish spots; and fifth, production of many mottled leaves.

Older plants of *Nicotiana tabacum* may not show these phases so clearly when infected. In general, the period between infection and clearing of veins is lengthened in older plants, especially if an older leaf is chosen for inoculation. Stunting is less marked and clearing of veins less conspicuous than in the younger plant, although sometimes taking the form of a partial blanching of a considerable number of leaves near the top of the plant. The first mottled leaves are much less distorted than are those of young plants. The period of production of comparatively normal looking, green leaves is inconspicuous and difficult to distinguish from the later period of the production of moderately mottled and slightly distorted leaves characteristic of the late infection in both young and old plants.

Nicotiana species responding similarly. The symptoms caused by infection with tobacco mosaic in *Nicotiana sylvestris* Spegaz. & Comes are almost identical with those described for *N. tabacum*, and those produced by *N. longiflora* Cav., *N. rusbyi* Britton, *N. suaveolens* Lehm., and *N. palmeri* A. Gray are similar. The symptoms on *N. trigonophylla* Dun. are of the same sort, but mottling and distortion are less pronounced.

Nicotiana tomentosa. Outgrowth of leaf tissue on the under side of mottled leaves, not previously described as a symptom of infection with tobacco mosaic, occurs regularly in *N. tomentosa.* In this plant yellowish primary lesions occur after inoculation. The systemic disease is characterized by some isolated secondary yellowish lesions on some leaves, and by severe mottling and distortion on others. The yellowed areas of mottled leaves sometimes expand fairly well, but occasionally very little. On the lower surfaces of diseased leaves leaf-like outgrowths occur around some of the areas of least expansion (Fig. 5). The outgrowths may extend a quar-

ter of an inch or more below the leaf lamina. All observed infected plants have shown some outgrowths sooner or later, although mottling and distortion have sometimes become well advanced before any sign of outgrowth has appeared. Healthy plants of *N. tomentosa* have never shown such an abnormality of growth.

Nicotiana paniculata. Nicotiana paniculata reacts to infection with tobacco mosaic virus as does *N. tomentosa*, forming leaf-like outgrowths of green tissue around the edges of poorly expanded yellow areas on the lower surfaces of severely distorted, mottled leaves.

Similar formation of leafy outgrowths was found by Storey (14) in Amani, Tanganyika Territory, as a result of infection by a non-mottling

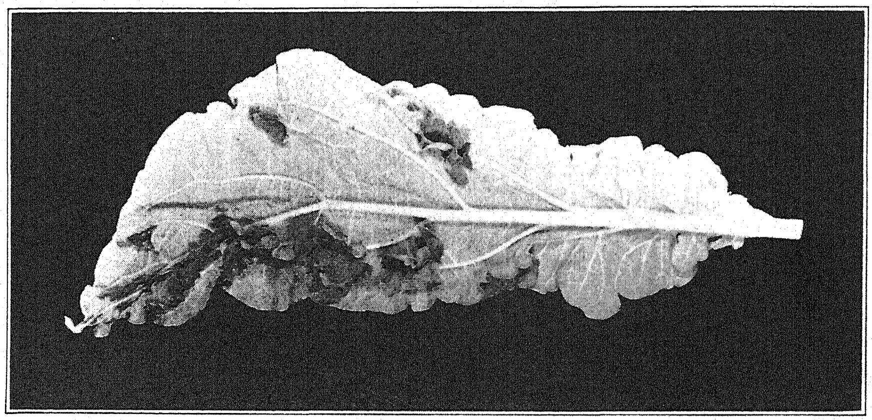

FIGURE 5. Distorted leaf of *Nicotiana tomentosa* showing leaf-like outgrowths on lower side.

virus on *N. tabacum*. He describes the condition in terms which seem to indicate a marked resemblance to the outgrowths associated with tobacco mosaic in *N. tomentosa* and *N. paniculata*, although the virus in his case appeared not to be that of tobacco mosaic since it did not mottle *N. tabacum*.

Species in other genera. Petunia varieties and *Proboscidea louisiana* Woot. & Stand. (*Martynia louisiana* Mill.) react approximately as does *N. tabacum*, with pronounced mottling and distortion of leaves. *Hyoscyamus niger* L. is severely stunted and yellowed after clearing of veins; it later produces leaves brilliantly mottled. *Lycopersicon esculentum* Mill. also produces a mottling and distorting infection, which is on the whole a little less severe than the corresponding mottling and distortion of *N. tabacum*. In *Solanum nigrum* distortion is slight, and the yellowish green and green areas of the mosaic pattern are little different in greenness. Young plants

of *Solanum melongena* var. Long White and var. Peking Green show obscure mottling on leaves forming at the time of the onset of the systemic disease; later these plants may develop nearly unmottled leaves and become almost like symptomless carriers.

Physalis peruviana. Physalis peruviana responds to infection with tobacco mosaic in an unusual way. Infected leaves rapidly produce large amounts of virus; they do not become much yellowed, nor do they show any tendency to fall off. Leaves nearer the top of the plant show a trace of clearing of veins some days after the plant is inoculated; subsequently formed leaves display an obscure mottling with small green blisters on a slightly lighter yellowish-green background. The virus content of these

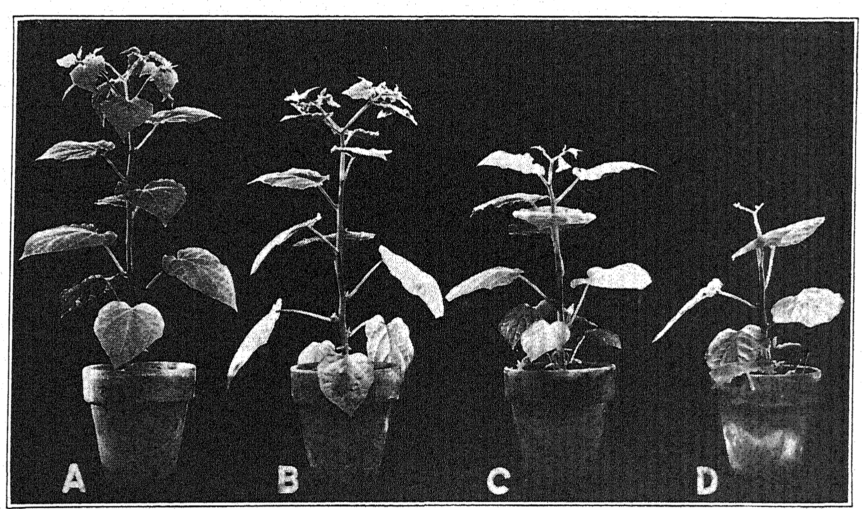

FIGURE 6. Plants of *Physalis peruviana*, showing degrees of defoliation. A, Uninoculated control plant; B, Infected plant which dropped a few partly expanded leaves; C, Infected plant which dropped many partly expanded leaves, but retained blossom; D, Infected plant which dropped all partly expanded leaves, and all blossoms; it retained only oldest leaves, including the one originally inoculated.

mottled leaves is high. Considerable defoliation occurs, and individual plants are affected differently, some losing all of their partly expanded leaves, others losing few or none (Fig. 6). Infected plants do not lose the older leaves, whether these are inoculated or uninoculated. The tiniest leaves at the tips of the branches are not dropped at first, but later may die. Blossoms are sometimes lost, sometimes not. Defoliated plants are slow to begin growth again, but finally send out shoots from axillary buds near the base of the plant. Many of these shoots become defoliated except for their youngest and oldest leaves; some begin to grow rapidly and pro-

duce conspicuously mottled foliage. *P. peruviana* differs from other species in which defoliation is reported in this paper, because it drops partly expanded leaves affected with the systemic disease, but does not drop the inoculated leaves even after these have long had a high virus content.

YELLOWING

There is a transitory yellowing of the leaves at the top of the plant in some hosts which have mottling as their chief symptom when infected with tobacco mosaic. A very severe and persistent yellowing, on the other hand, characterizes infected plants of *Nicotiana multivalvis*, *N. quadrivalvis*, *N. clevelandii*, and many *Capsicum frutescens* varieties. This yellowing or blanching, although followed by mottling if the plant survives long enough, is characteristically so conspicuous and enduring as to be considered the chief feature of the complex of symptoms shown by such plants.

Nicotiana multivalvis. Inoculation of young *Nicotiana multivalvis* plants in the rosette stage is followed after a few days by clearing of veins, with subsequent yellowing of all recently formed leaves. Persistent reduction of the growth rate of the plant occurs. Uninoculated plants of the same lot may form a flower stem and begin to blossom before the infected plants produce any appreciable new leaf tissue. In this stage of the infection the plants often die, especially if the older leaves at the base of the plant do not persist to furnish food until growth can be resumed at the top of the plant (Fig. 7 A to D). After an extended period of yellowing and stunting which may last from one to five weeks, renewed growth results in the formation of mottled and distorted leaves, or of narrow leaves in which yellowed tissue fails to develop and only the green areas expand to appreciable width of lamina. Plants in this condition may eventually die in the rosette stage.

Inoculation of older plants, in which flowering has already begun, is followed by a gradual yellowing of much of the foliage, beginning with the bases of the most recently developed leaves, and later spreading to all portions of these leaves and of other older leaves. Blossoming ceases soon after the appearance of yellowing at the top of the plant. Blossom buds still develop for some days, and appear ready to open, but do not. Upon close examination, the white tissues of the blossom buds are seen to be discolored, the flower stems show some buds dying, and abscission occurs sometimes at a touch. Abscission is not nearly so characteristic of this species as it is of the *Capsicum frutescens* varieties discussed in another section, not affecting leaves or fruit in this species. Very young flower buds frequently die and drop off while still small, and finally no more flowers appear. Seed pods which are completely formed before the yellowing of leaves in the upper part of the plant takes place persist and ripen their

seeds. Pods partly formed at the time of appearance of the systemic disease are sometimes distorted, ripening seeds on one side of the pod but not on the other side. The yellowed plants, no longer producing new flowers or foliage, form a striking contrast to the uninoculated plants, which are characterized in this species by smooth, dark green foliage, white blossoms, and large seed pods. The yellowed plants are similar in appearance to plants affected by the yellows diseases, such as aster yellows and peach

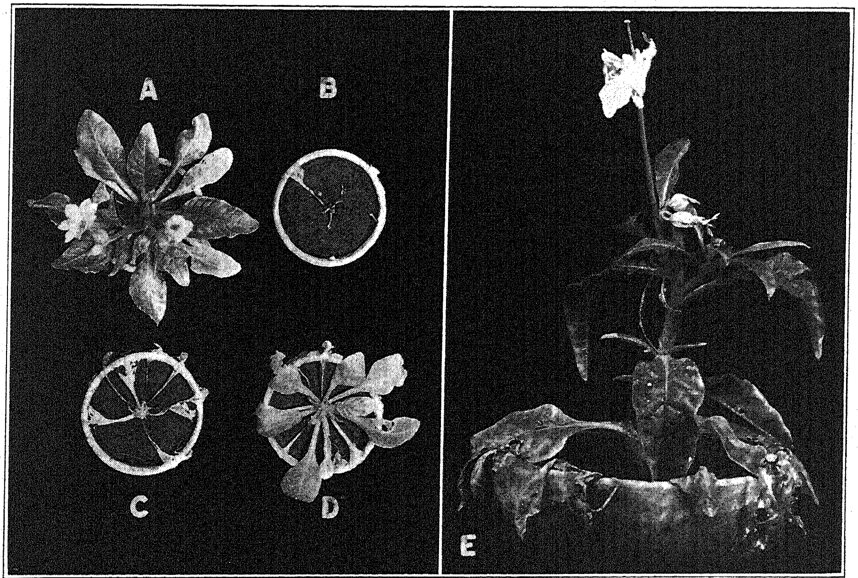

FIGURE 7. Plants of *Nicotiana quadrivalvis*. A, Uninoculated control plant; B to D: Plants of same age infected when young. B, Dying; C, Yellowed, only small center leaves alive; D, Yellowed, except oldest leaves; E, Plant inoculated when at blossoming stage; photographed after long period of yellowing and stunting, showing recently produced narrow, mottled, distorted leaves, and distorted blossom.

yellows, but show distinct differences from such diseases in their failure to force dormant buds, in subsequent mottling of leaves, and in a normal response to gravity.

Many of these plants infected when comparatively old also die in a short time, perhaps partly because of starvation from lack of green tissue. Others survive long enough to send out narrow, distorted, mottled leaves, consisting mostly of green tissue, the yellow areas expanding very little. The new shoots may bear distorted flowers (Fig. 7 E), which produce seed pods containing some seeds. All plants grown from such seeds have proved healthy.

Nicotiana quadrivalvis and *N. clevelandii* resemble *N. multivalvis* in their yellowing symptoms although *N. clevelandii* becomes a little less intensely yellowed.

Capsicum frutescens. Yellowing is a very prominent symptom of tobacco mosaic in most varieties of garden pepper, although followed by mottling in the course of time. It is often associated with leaf abscission without necrosis, a phenomenon not previously reported as a result of infection with tobacco mosaic virus. A few varieties of pepper have only necrotic symptoms (7, p. 169, Fig. 3 J); these are Tabasco, Anaheim Chili, and Creole among the *Capsicum frutescens* varieties, and a species grown under the name of *Capsicum minimum* Blanco. A few show little or no yellowing preceding mild mottling; such are Sunnybrook Cheese and Long Red Cayenne. Among those showing conspicuous yellowing are Neapolitan, Red Chili, Mammoth Golden Queen, Red Cherry, Sweet Mountain, Sweet Meat Glory, Large Bell, Pimiento, Early Giant, Golden Dawn, Rainbow, and Ruby King.

In young plants of the varieties showing a yellowing response, yellowish primary lesions similar to those described on *Nicotiana tabacum* (7) appear on the third or fourth day after inoculation and mark spots in which starch is disturbed, as in *N. tabacum*. Sometimes inoculated leaves, if old, yellow so rapidly that primary lesions are much greener than the unaffected parts of the leaves (Fig. 8 A). Thus yellowish primary lesions on green leaves or green local lesions on yellow leaves may appear, according to the condition of the leaves at the time of inoculation. Abscission of the leaves, when it occurs, apparently does not prevent systemic infection, which appears soon as yellowing of young leaves at the tips of branches. Severe stunting commonly occurs. The yellowing lasts longer than the transient yellowing in *N. tabacum*, but disappears largely by the end of the third week after inoculation. It is replaced by a greening of the previously blanched tops and by the gradual development of moderately mottled and distorted leaves. Old leaves invaded by virus after they are fully expanded sometimes show oak-leaf patterns in green as they lose their chlorophyll from age (Fig. 8 B and C).

Inoculation of older plants late in the season, at the time of first ripening of fruits, results in a condition which greatly emphasizes the yellowing and leaf-dropping, but causes little stunting since the plants are already nearly full size. Leaves at the tips of inoculated branches turn conspicuously yellow, corresponding leaves of other branches of the same plants remaining green. The infected branches then often defoliate almost entirely and also drop blossoms and young fruit, retaining only the smallest leaves. These leaves do not develop for some time. Such extreme defoliation has not previously been recognized as a symptom of infection with tobacco mosaic virus. The loss of blossoms and fruits has also not been

described previously in this connection. If the season is not too far advanced the small leaves and buds may finally develop and display mottling, but the advent of cold weather often interferes, with the result that conspicuous yellowing and defoliation of branches are the only symptoms expressed.

Low temperatures cause certain pepper varieties which normally produce yellowish primary lesions on inoculated leaves to produce necrotic

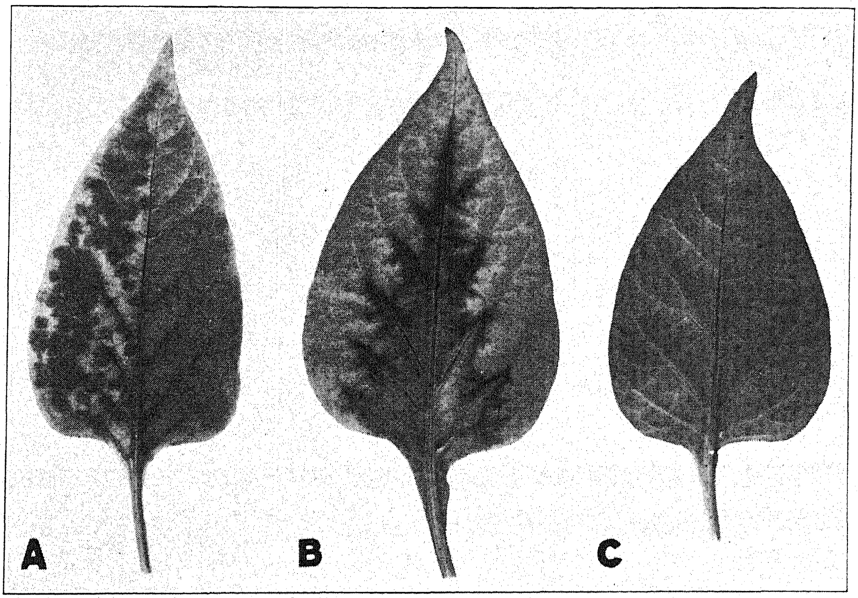

FIGURE 8. Leaves of Pimiento pepper, showing chlorophyll retention patterns in leaves yellowing with age. A, Primary lesions, 5 days after inoculation by rubbing half of leaf; B, Pattern of systemic disease in a leaf already expanded at time of inoculation; C, Yellowed leaf of healthy plant.

lesions only. In the systemic disease in cold autumn weather necrotic oakleaf patterns may be found on old leaves invaded by the virus, although at higher temperatures such leaves would remain green and unmarked by necrosis, displaying the virus pattern only as irregularities in starch content of infected leaves.

Yellowing is a conspicuous part of the symptoms of most of the pepper varieties, and occurs on the majority of the kinds commonly grown. The commonest type of large-fruited garden peppers, such as Large Bell, Pimiento, and Early Giant, are much yellowed before mottling, and in late season infections may show yellowing without the development of later mottling.

Little has been published on the effect of the specific virus of tobacco mosaic on peppers. Previous reports have been concerned with "pepper mosaic" in the field and have perhaps referred to injury caused by the presence of more than one mosaic virus. It seems appropriate therefore to describe the damage caused by tobacco mosaic virus alone when it oc-

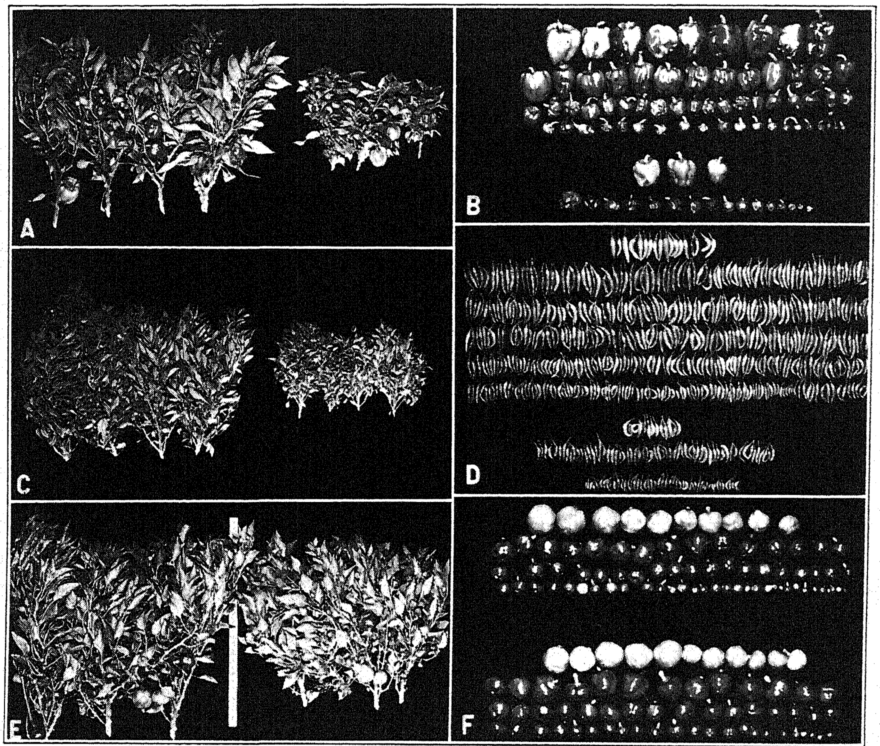

FIGURE 9. A, Four healthy and four mosaic plants of Large Bell pepper; B, Fruit of these plants (healthy above, diseased below), showing extent of injury to crop; C and D, Same, Red Chili pepper; E and F, Same, Sunnybrook Cheese pepper, less reduction in size of plants, and little or no harm to crop of fruit.

curs on pepper varieties. Varieties showing severe yellowing and stunting produce little or no crop of fruit; those showing mild yellowing and stunting may furnish some crop; and a few varieties, such as Sunnybrook Cheese and Long Red Cayenne, which show little injury to the foliage, may furnish a good crop. Reduction of crop results partly from abscission of flowers and young fruits and partly from long delay in sending out new foliage after the onset of the disease.

In Figure 9 A four healthy plants of average size of the variety Large

Bell are shown in contrast to four stunted mosaic plants of the same age. The fruits of these plants are shown in Figure 9 B. Red fruits constitute the first line in each set; the fruits of the diseased plants shown below the fruits of the healthy plants manifestly represent but an insignificant part of a normal crop.

Somewhat less severe harm to the crop is shown in the case of Red Chili pepper in Figure 9 C and D; yet in the diseased set of fruit very few specimens are as large or as smooth as the fruits of the healthy plants.

Much less reduction of foliage is shown in Figure 9 E for the more nearly passive variety Sunnybrook Cheese, although the diseased plants are shorter than the healthy ones. The fruits from these plants shown in Figure 9 F indicate that this variety is actually hastened in maturity by the onset of the disease, the immature fruits of the diseased plants being fewer, and the nearly mature fruits being satisfactory in number, size, and appearance.

Limited surveys in the field in New Jersey during 1931 disclosed the presence of tobacco mosaic in commercial plantings and indicated that considerable loss occurs from this disease. It was also found that at least one other similar mosaic disease occurred in the pepper fields; its virus produced no necrotic lesions on *Nicotiana glutinosa*, but a delayed, mild mottling of that species.

NATURAL IMMUNITY IN SOLANACEOUS PLANTS

Although many species of solanaceous plants are susceptible to infection with tobacco mosaic virus, as illustrated by the fact that no species of the genus *Nicotiana* is altogether immune to infection so far as is known, there are some species of the Solanaceae which have appeared to be naturally immune, especially in genera not very closely allied to those found most susceptible. This is of interest because it is not yet known whether immunity bears any close relation to the symptom groups which have been discussed. There seem to be evidences in the behavior of plants described above of tendencies in several directions toward immunity. Thus, plants may become infected easily and yet almost inhibit the formation of virus, as seems to be the case in *N. glutinosa*; they may allow virus to spread erratically from the inoculated leaf, as in *N. glauca*, or localize it consistently, as in all the plants treated as showing typical localized necrosis; they may become infected by mechanical inoculation less readily than other species, but produce plentiful amounts of virus when infected, as is perhaps true of such plants as peppers infected by handling in planting. Thus it may be said that in solanaceous plants susceptible to tobacco mosaic infection there may be discerned tendencies of three kinds toward immunity: (a) a tendency opposing infection at time of inoculation; (b) a tendency opposing increase of virus after infection; and (c) a tendency op-

posing spread of virus. These might be spoken of briefly as partial resistance (a) to infection by virus, (b) to increase of virus, and (c) to spread of virus.

It is a question whether the natural immunity of some solanaceous species and the natural immunity supposed to occur in most species of other families to infection by this virus is the result of more pronounced manifestation of one or more of these tendencies to resistance, or is of some other nature.

Physalis viscosa L. A very interesting condition occurs in the genus *Physalis*. One species, *P. angulata*, produces a systemic necrosis; another, *P. alkekengi*, may act as a symptomless carrier; *P. peruviana* becomes systemically infected and displays obscure mottling and defoliation; but *P. viscosa* has given no indication of susceptibility to infection thus far.

Physalis viscosa was repeatedly inoculated with both light and heavy inoculations, and yet never showed the presence of any virus in leaves at the top of the plant, never showed any symptoms, and never showed an increase of virus in the inoculated leaf. Virus which could at first be detected on the inoculated leaf because of the excess of virus used in the attempt at inoculation disappeared after some days. Finally a branch of this plant was grafted on an infected plant of *Physalis peruviana*, which contained much virus; no symptoms except slight yellowing resulted in *P. viscosa*, and on testing the leaves and stem of the cion for virus content it was found that no virus could be demonstrated. This evidence indicates that *P. viscosa* is highly resistant to infection.

It would be a matter of interest to know whether the comparatively widespread immunity to infection in plants of other families is of the same nature as that which protects apparently immune solanaceous species. It would also be interesting to know whether more careful search would not disclose some species of many other families which could serve as hosts of this virus, since *Phaseolus vulgaris* in the Leguminaceae (12) and *Martynia louisiana* in the Martyniaceae (3, p. 468) are recognized as hosts. It seems likely that tobacco mosaic virus may infect and multiply in hosts in which its presence has not yet been recognized. This is especially likely since recent studies have shown that responses such as leaf abscission, localized necrosis, abnormal tissue outgrowths, and death of young plants can be recognized among the symptoms caused by this virus, and since some of its present hosts are known to be masked carriers. Methods are at hand to detect and measure on successive days after inoculation the increase of virus in inoculated leaves and other parts of plants which may be susceptible to infection, which may be able to allow multiplication of virus locally or systemically, but which may show no visible symptoms of the presence of the virus.

SUMMARY

Descriptions are given of primary and secondary symptoms produced as a result of infection by virus of the common field type of tobacco mosaic in a number of different plant species belonging to the genera *Nicotiana*, *Solanum*, *Physalis*, *Capsicum*, *Lycopersicon*, *Datura*, *Petunia*, *Nicandra*, *Lycium*, *Hyoscyamus*, *Martynia*, and *Phaseolus*.

Symptoms not previously described as characterizing infections with tobacco mosaic virus are prolonged yellowing, leaf abscission, flower and fruit abscission, outgrowth of tissue on the lower side of mottled leaves, death of plants by systemic necrosis, failure to remove chlorophyll in tissues recently invaded by virus, bending of upper stem toward side of plant bearing inoculated leaf, and intensification of pigment in spots on flowers.

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ACQUIRED IMMUNITY TO RING-SPOT IN NICOTIANA

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A number of workers have reported recovery of plants from diseases caused by viruses. It is generally believed, however, that such plants are not immune but are susceptible to reinfection with the virus of the disease from which they recover. The susceptibility of recovered plants has been clearly demonstrated for certain of these diseases. Although some evidence to the contrary has been obtained, most pathologists hold to the view that acquired immunity in plants, comparable to acquired immunity in animals, has not been definitely proved. The behavior of ring-spot, a virus disease of tobacco, is, in many respects, unlike that reported for most other virus diseases. Some species of plants invariably recover from ring-spot and do not develop symptoms when reinoculated with the virus of this disease. In a series of investigations on ring-spot, Wingard (45) observed that a number of different kinds of plants recovered and produced apparently healthy leaves. He also found that the expressed sap from these leaves was highly infectious when applied to healthy plants and stated that he was unable to obtain infection on the recovered portions of such plants. These observations led him to suggest that the plants developed an immunity to the disease.

Because of the opportunity to obtain information on acquired immunity offered by a study of ring-spot, the unusual behavior of this disease has been investigated by the writer. The purpose of this paper is to present the experimental results which were obtained.

REVIEW OF LITERATURE

The attention of pathologists has been directed to the question of immunity for many years. A study of the phenomena accompanying active and passive immunization of animals has yielded considerable information regarding the factors involved in these processes. From this study it is well recognized that immunity is not dependent upon one but upon many factors. The results of a number of investigators have shown that the phagocytic theory of Metchnikoff is not adequate to account for immunity in animals. It was first shown by Nuttal (30) that defibrinated blood had a bactericidal action. Buchner (7) confirmed these results and showed further that this bactericidal action was present in the cell-free serum of clotted blood. Shortly after this discovery, von Behring and Kitasato (2) were able to show that animals could be immunized with the products of bacterial metabolism and that this immunity was associated with the

[1] The writer is indebted to Professors S. F. Trelease and R. A. Harper of Columbia University and to Dr. L. O. Kunkel and other members of the staff of the Boyce Thompson Institute for suggestions and criticisms made during the course of these investigations.

presence of specific antibodies in the treated animal. These results were later extended until it is now generally understood that specific antibodies occur in the blood or blood serum of animals injected with practically any foreign protein.

The search for antibody formation in plants was a logical outgrowth of the discovery of the existence of such substances in immunized animals. The existence of normal agglutinins, precipitins, and lysins in plants has been demonstrated by a number of workers but it has not been definitely shown that these are the active agents which determine immunity against specific organisms. As early as 1911, Bernard (4) showed that sap of orchids contained material which had a fungicidal action against certain mycorrhizal fungi. Kriteschewsky (27) found that normal agglutinins against *Bacterium typhis* and *Vibrio cholerae* were present in the juice of *Cotyledon scheidekeri* and that such agglutinins were thermostabile and non-specific. Wagner (44) obtained bactericidal material from tubers of *Solanum tuberosum* and from leaves and roots of *Sempervivum hausmanii* and *Beta vulgaris*. Similar results were obtained by Berridge (5) who showed that the fresh juice of potato tubers had an agglutinating and plasmolytic action on many non-pathogenic species of bacteria at the normal hydrogen ion concentration of the cell sap but did not react with any of the pathogenic organisms tested at the same hydrogen ion concentration.

It has been shown by Kostoff (26) that extracts of certain species of the Solanaceae exhibit precipitin and lytic reactions when added to extracts of other related species. He has shown further that the precipitation capacity of the extracts is increased by intergrafting. This increase in potency of the precipitin reaction was regarded by Kostoff as an expression of acquired immunity. Chester (9) found that a similar precipitin reaction occurred between an extract of lilac scions previously grafted on privet stock and an extract of normal privet. He also showed that such precipitins could be induced by growing lilacs under unfavorable soil conditions. From a chemical study of the precipitin reaction in 42 species of solanaceous and woody plants, Chester (10) concluded that four different reactions were involved. The majority of the precipitates consisted of calcium oxalate which was formed when an excess of calcium ion was present in one of the two plant juices, an excess of oxalate ion in the other. Three other groups of precipitates were distinguished but the nature of these has not yet been fully determined.

The apparently low degree of specificity of the precipitins, agglutinins, and lysins developed in plants is sharply contrasted with the high degree of specificity demonstrated for animal antibodies. It is believed that the production of specific antibodies in plants has not been conclusively demonstrated and it remains for further work to demonstrate the importance of these precipitins and agglutinins in plant immunity.

Attempts have been made to induce immunity in plants by inoculating them with virulent or attenuated cultures and also by injection with products of bacterial metabolism. As early as 1903, Hiltner and Störmer (19) reported that legumes infected with a virulent root nodule organism could not be reinfected with a less virulent one. A similar conclusion was reached by Dunham and Baldwin (13). A number of European investigators have reported successful or partially successful immunization of plants against various pathogenic organisms by exposing them to attenuated cultures or to extracts of pathogens. This work has been summarized recently by Carbone and Arnaudi (8). Although the evidence reported by these writers indicates that the treated plants were more resistant to attack than were untreated controls, it is not clear from this evidence whether the increased resistance was due to a specific reaction against the pathogen or to the poor growth made by the treated plants.

It has been shown by other workers that plants grown under certain environmental conditions are more resistant to some diseases than those grown under other conditions. Spinks (37) observed that resistance of wheat to rust and of barley to mildew was increased by supplying the plants with large amounts of potash whereas resistance was decreased by addition of considerable quantities of available nitrogen. Reed (34) was unable to obtain infection with powdery mildew in barley and wheat plants kept in the dark. Thomas (40) reported that susceptibility of celery plants to *Septoria apii* was increased by fertilization with nitrates and decreased by addition of calcium sulphate. It has been shown by Dickson and Holbert (12) that wheat seedlings inoculated with *Gibberella saubinetti* blighted at high temperatures and remained healthy when grown at temperatures below 10° C., whereas corn seedlings infected with the same fungus exhibited an opposite reaction. Trelease and Trelease (42) observed that wheat plants supplied with high nitrogen were less resistant to mildew than those supplied with high phosphorus or potassium. Stakman and Aamodt (38) concluded that the injury caused to wheat plants by the rust fungus was distinctly increased by heavy fertilization although the degree of physiologic susceptibility was apparently not changed.

Virus diseases. Plants of several different species have been observed to recover from diseases caused by viruses. It is generally believed, however, that such plants are not immune to reinfection. Probably the earliest report of recovery from a virus disease of plants was that of Baur (1) who observed that *Abutilon thompsoni* plants grown in shade frequently recovered from mosaic. He also found that such plants could be reinfected by grafting with diseased material. In the same paper, Baur reported that abutilon plants infected with mosiac occasionally developed healthy shoots and that, although such shoots could not be used to infect other plants, they were immune to reinfection. His evidence for immunity to reinfection,

however, is not convincing and the observations have not been confirmed by other workers.

Several workers have reported that certain varieties of sugar cane frequently recover from mosaic but they believed that the recovered portions of the plants were free from virus and could be reinfected. In 1920, Brandes (6) reported recovery of sugar cane and sorghum from corn mosaic. Kunkel (28) found that, in one case, a recovered sugar cane plant again showed mosaic symptoms and he also observed that plants grown from cuttings of recovered sugar cane plants appeared healthy but occasionally mosaic symptoms reappeared after several months. He believed that such plants were healthy and that the reappearance of symptoms was due to new infections. Storey (39) found that sugar cane plants often recovered from the streak disease of corn. Recovery of sugar beets from the mosaic disease was reported by Robbins (35) who also suggested that the recovered plants might still serve as a source of infection. Johnson (22) observed recovery of tobacco plants from cucumber mosaic and found that the recovered plants were carriers of virus. He did not determine whether it was possible to reinfect the recovered plants. Similar results were obtained by Porter (31) who found that the Chinese Long variety of cucumbers occasionally recovered from mosaic and that the recovered plants contained mosaic virus.

It has been observed that symptoms on plants infected with mosaic or other viruses gradually disappear when such plants are placed under special environmental conditions. This gradual disappearance of symptoms on diseased plants under certain conditions is referred to as masking of symptoms. Plants in which the symptoms have become masked again develop typical symptoms when returned to their original environment. Abnormal temperature conditions are known to cause masking of symptoms of some diseases. Goss and Peltier (16) observed that the symptoms of rugose mosaic of potatoes were masked when the plants were kept at air temperatures above 25° C. while the symptoms of spindle tuber and yellow dwarf of potatoes were masked at low temperatures. Johnson (23) showed that the symptoms of tobacco mosaic were masked at 36° to 37° C. Bennett (3) found that the symptoms of red raspberry mosaic and of mild mosaic were masked at 28° C. but that the symptoms of curl were not masked at a wide range of temperatures.

Some plants are known to carry viruses without showing any marked symptoms of disease at any temperature at which they have been grown. Such plants are known as masked carriers. Nishimura (29) showed that *Physalis alkekengi* is a masked carrier of tobacco mosaic. Thrupp (41) reported that "M45," a variety of hops, was found to be a symptomless carrier of hop mosaic. Johnson (24) showed that healthy-appearing pota-

toes carry a virus which produces ring-spot symptoms in tobacco plants when infected with this virus.

In the first part of this review, reference was made to some of the phenomena following immunization against diseases in general. It might be well at this time to refer to some of the phenomena accompanying immunization of animals against virus diseases. With few exceptions, animals that recover from virus diseases have a lasting immunity. Although it has not been definitely proved, many investigators regard this immunity as being associated with the presence of active virus in the recovered animal. Considerable evidence has been obtained to show that virus may persist in immune animals. Cole and Kuttner (11) reported that old animals were carriers of the salivary gland virus of guinea pigs but that virus was not obtained from pigs less than one month old. They found that this virus could be obtained at any time from a pig once infected and that it was highly infectious for pigs not previously infected. Kock (25) found that the virus of infectious anemia was present in the blood of horses as long as seven years after an attack of this disease.

Ring-spot. Tobacco ring-spot was observed as early as 1917 in the tobacco fields of Virginia. The infectious nature of the disease was first demonstrated by Fromme, Wingard, and Priode (15). These writers obtained infection by rubbing or injecting with juice secured from leaves and stems of diseased plants. The disease was transferred to five species of *Nicotiana* including a number of varieties of *N. tabacum*. They suggested that ring-spot should be classified with the virus group of diseases.

Priode (33) succeeded in transferring ring-spot to *Beta vulgaris* L., *Phytolacca decandra* L., *Petunia hybrida*, and *Tetragonia expansa* Murr. He showed further that local lesions were produced at the points of entrance of ring-spot virus into leaf tissue of tobacco plants. These local lesions occurred as rings of necrotic tissue around pin point punctures. Stems of tobacco plants, inoculated by pin punctures, did not develop lesions although the plants later developed systemic symptoms of ring-spot. Priode was unable to obtain infection with juice filtered through Berkefeld filters of the N grade but found that unfiltered juice was highly infectious after storage at $-5°$ C. for 85 days. On the other hand, diseased leaf tissue proved to be non-infectious after drying. In a test involving 600 seedlings, Priode found that the virus of ring-spot was not seed-transmitted.

Wingard (45) studied the host range of tobacco ring-spot and succeeded in transferring it to 38 genera of plants belonging in 17 different families but was unable to obtain infection in plants of 34 other genera. Wingard was apparently the first to observe recovery from tobacco ring-spot. He found that ring-spot plants, after displaying characteristic necrotic spots

for a period of 10 to 14 days following inoculation, finally developed leaves which appeared healthy or showed only a faint grayish mottling. The juice from such leaves proved to be infectious when applied to leaves of previously healthy plants. Wingard stated further that attempts to reinfect the recovered portions of such plants by inoculation with virulent juice were unsuccessful.

Johnson (22) reported seed transmission of a ring-spot virus through seed of tobacco in a few instances. Henderson (17) concluded that ring-spot virus was transmitted through seed of petunia in about 20 per cent of the seedlings.

Henderson and Wingard (18) reported the results of further work on recovery from ring-spot and concluded that the symptoms remained masked for over a year in serial cuttings made from recovered tobacco plants. They also studied the properties of ring-spot virus and found that it remained infectious when stored for 22 months at $-18°$ C., but that it became non-infectious when kept at room temperature for only a short time. These writers showed further that the virus resisted heating for ten minutes at 60° C., but lost its infectious property when heated for three minutes at 70° C. The virus was found to pass Berkefeld filters of the V, N, and W grades if first freed from the larger particles of the juice in which it was present. Studies on seed transmission in a test involving 64,500 seedlings yielded negative results.

Fenne (14) reported that the tobacco fields kept under observation during the summer months showed a higher percentage of infection with ring-spot in the early part of the season than at a later date and attributed this decrease to masking of symptoms in many of the infected plants.

Valleau (43) reported seed transmission of two ring-spot diseases of tobacco in proportions up to 15 per cent. He does not state whether either of these two diseases is identical with that studied by Priode and by Henderson and Wingard.

It is well to point out at this time that there are other ring-spot diseases which are apparently distinct from tobacco ring-spot. It has been reported by Johnson (24) that healthy-appearing potatoes carry a virus which also attacks tobacco and produces ring-spot symptoms. A comparison of the symptoms produced by this disease with those of ring-spot has shown that the two are not identical (18). Smith (36) distinguished three separate viruses which attack solanaceous plants and cause the development of ring-spot symptoms. The diseases produced by these viruses are believed to be similar to but distinct from the ring-spot disease with which this paper is concerned.

MATERIALS AND METHODS

The virus used in the studies reported in this paper was obtained from Dr. S. A. Wingard[2] of the Virginia Agricultural Experiment Station. It

was originally secured from a diseased plant in a commercial tobacco planting in Virginia.

Except where otherwise noted, ring-spot was transferred to test plants by means of the rubbing method. Diseased leaf or stem tissue was wrapped in cheesecloth squares, macerated by pounding with a new pot label, and rubbed over the upper surface of one or more leaves. When diluted juice was used as inoculum, small cheesecloth pads, saturated with the diluted juice, were rubbed over the leaves. Immediately after inoculation, the leaves were flushed with water in order to remove materials which might cause burning of the tissue. Stem inoculations were made by puncturing the stems of test plants with No. 00 insect pins previously dipped into infectious juice. Grafting and budding methods were also employed in transmitting ring-spot. A simple type of whip graft was used in the grafting experiments.

Cuttings from healthy and recovered plants were propagated by growing them in a mixture of moist sand and peat moss. Usually only tip cuttings were used but occasionally more than one cutting was grown from a single plant. After three to four weeks, a good root system had developed on the cuttings and they were then transferred to sterilized soil in four-inch pots.

Holmes (21) found that although visible lesions did not always appear on leaves of Turkish tobacco plants inoculated with mosaic virus, the presence of this virus in leaves considerably delayed the removal of starch from invaded areas. This delay was demonstrated by keeping infected plants in darkness for several hours and subsequently staining them in iodine. The method described by Holmes was used by the writer to study the tendency of ring-spot virus to delay removal of starch from tissue of Turkish tobacco which it had previously invaded.

A method of measuring the concentration of virus in mosaic tobacco plants has been described by Holmes (20). He observed that more lesions developed on leaves of *Nicotiana glutinosa* inoculated with undiluted tobacco mosaic virus than on similar leaves inoculated with diluted samples of this virus. Price (32) obtained similar results when leaves of certain varieties of the common bean, *Phaseolus vulgaris*, were inoculated with various dilutions of tobacco mosaic virus. It was pointed out that the advantage of beans as test plants results from the rapidity with which they grow.

Studies were conducted in order to obtain a similar method for measuring ring-spot virus concentration. A number of beans and cowpeas were tested to determine whether necrotic lesions would develop on leaves of these plants after inoculation with ring-spot virus. The primary leaves and first compound leaves of 4 to 12 plants of each of these varieties were

[2] The writer wishes to take this opportunity to thank Dr. Wingard for his kindness in supplying the sample of virus used in this work.

rubbed with undiluted extract of diseased plants. Controls consisted of uninoculated plants grown under the same environmental conditions. Local lesions developed in two or three days after inoculation on the primary leaves of the following varieties of the common bean (*Phaseolus vulgaris* L.): Cut Short or Corn Hill, Davis Kidney, Early Golden Cluster, Early Red Valentine, Early Refugee, Great Northern, Hodson Long Pod, Ideal Market, Improved Black Wax, Keeney's Stringless Refugee, Kentucky Wonder, Webber Wax, and White Creaseback. Local lesions developed on leaves of three varieties of *Phaselous lunatus* L.: Henderson's Bush Lima, Henderson's Improved Bush Lima, and Sieva. Similar lesions developed on inoculated leaves of three varieties of *P. lunatus* var. *macrocarpus* Benth.: Carpinteria, Extra Early Jersey, and King of the Garden. Local lesions developed on four of the varieties of cowpeas (*Vigna sinensis* Endl.) inoculated with ring-spot virus: Black, Early Buff, Groit, and New Era. The local lesions produced by inoculation with ring-spot virus were usually sharply delimited necrotic spots.

A test was made in order to determine whether ring-spot virus could be obtained from the local lesions which appeared on cowpea leaves inoculated with this virus. A small section of the leaf including a necrotic lesion was macerated between the tips of two new pot labels and rubbed over leaves of a healthy tobacco plant. A total of 18 of the necrotic lesions on Black cowpea leaves were tested in this manner. All 18 tobacco plants inoculated with juice from necrotic lesions developed typical ring-spot symptoms. Six control plants inoculated in a similar manner with juice from healthy cowpea leaves did not develop ring-spot symptoms. These results show that ring-spot virus was present in Black cowpea leaves on which necrotic lesions developed but was not present in any of the healthy leaves tested.

Systemic symptoms appeared in about six days on many of the varieties of beans and cowpeas which were inoculated with ring-spot virus. These symptoms consisted of circular necrotic spots on the young leaves and irregular reddish lesions on the stems and leaf petioles. The reddish lesions were numerous and eventually caused the death of affected plants.

It appeared from these results that a number of varieties of beans and cowpeas might be used as test plants to measure ring-spot virus concentration. The writer selected the cowpea for this purpose because the leaves of this plant are thick and not easily injured in handling and also because the lesions which develop on inoculated leaves of this host, being large and distinct, are easily counted. The Black variety of cowpea was selected for use in all subsequent experiments in which it was desired to measure virus concentration.

In order to determine whether progressively fewer lesions develop on cowpea leaves as decreasing concentrations of ring-spot virus are used as

inoculum, a dilution experiment was conducted with a virus sample obtained from diseased Turkish tobacco plants. The results of this experiment are shown by the curve presented in Figure 1. Each point on the curve represents the average number of lesions per leaf resulting from inocula-

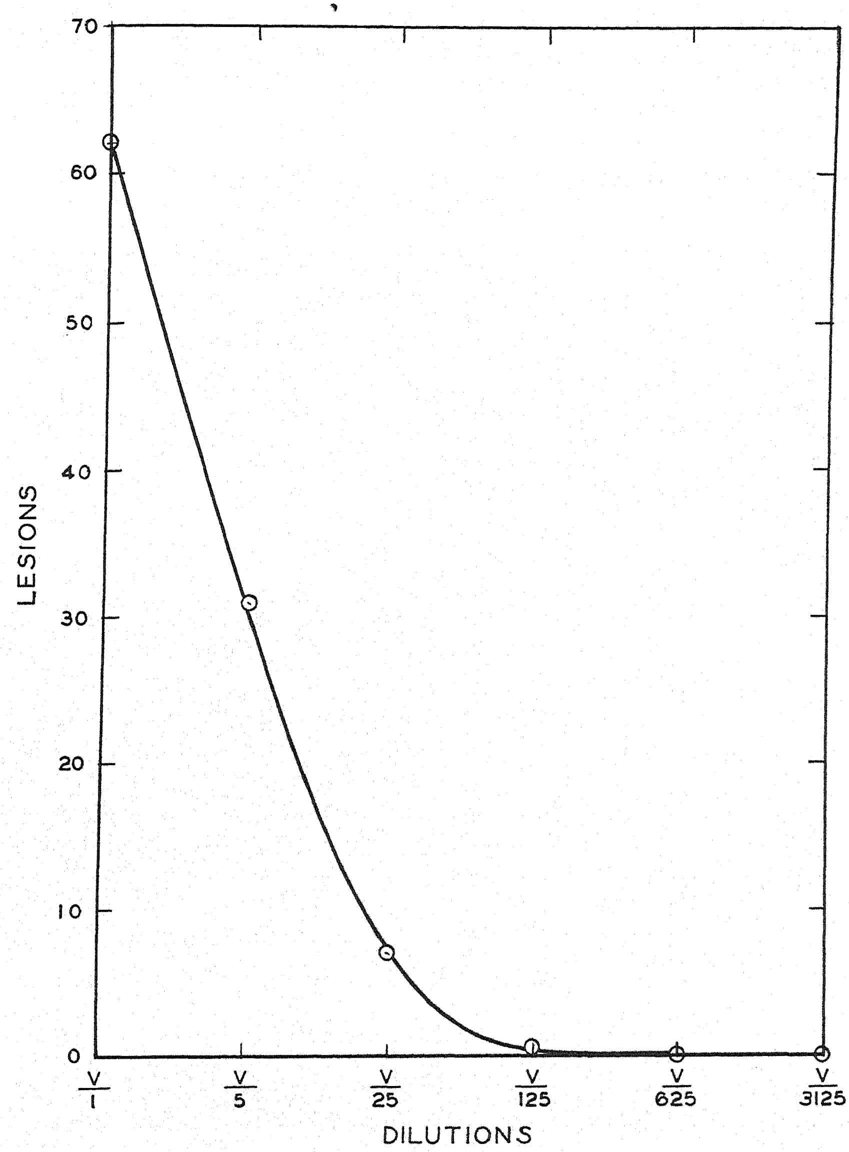

FIGURE 1. Effect of diluting ring-spot virus when *Vigna sinensis* var. Black is used as a test plant. The points on the curve represent the average number of lesions on each leaf produced by inoculation of 80 leaves of the test plant with the virus dilution indicated.

tion of 80 cowpea leaves with the virus sample indicated. The cowpea plants were of the same age and were grown under the same environmental conditions. As the dilution curve in Figure 1 indicates, more lesions appeared on leaves inoculated with undiluted juice than on a similar number of leaves inoculated with diluted juice. Although the curve indicates a high correlation between the average number of lesions per leaf and the concentration of virus in the inoculum, considerable variation was observed in the numbers of lesions which developed on different leaves inoculated with the same virus sample. The factors which are responsible for this variation have not been investigated sufficiently by the writer to warrant any definite conclusion regarding them. It may be that such factors as age of test plants and environmental conditions under which the test plants are grown influence the numbers of lesions produced by inoculation with a given sample of virus. It is hoped that a considerable improvement of the method can be made after a more thorough investigation of such factors.

Measurements of ring-spot virus concentration reported in this paper were made by inoculating 16 leaves of Black cowpea plants with the virus sample tested. The test plants were grown in a greenhouse in which the temperature was not allowed to fall below 21° C. Only plants that had not completely expanded their primary leaves at the time of inoculation were used.

DESCRIPTION OF THE DISEASE

This ring-spot disease of tobacco is known to occur on many different species of plants. Wingard (45) has given a description of the symptoms produced on a large number of species and it is therefore considered unnecessary to present a detailed description of the disease in this paper. A brief review of the most important symptoms and characteristics on species of *Nicotiana* will be given here. Four different phases of the disease can be distinguished: first, the primary lesions which appear soon after inoculation at the points where infections in leaf tissue were obtained by inoculation; second, similar systemic lesions which are produced on the young uninoculated leaves; third, the wavy lines of chlorotic or necrotic tissue which develop when the plants begin to recover; and, fourth, the healthy-appearing leaves characteristic of recovered plants.

Primary lesions develop three or four days after inoculation and consist of circular necrotic spots or of zonate lesions composed of rings of chlorotic or necrotic tissue alternating with rings of healthy-appearing tissue.

Systemic symptoms appear in six or more days after inoculation and are similar to the primary symptoms. The systemic lesions are generally larger than the primary lesions especially if they are centered on the larger

veins. They display the Liesegang phenomenon and tend to follow and encircle the veins. A previously undescribed symptom of ring-spot is clearing of the veins. This symptom has been observed frequently on some species, such as *N. quadrivalvis* Pursh and *N. quadrivalvis* var. *multivalvis* Gray, and rarely on others. The writer has never observed it on inoculated leaves but only on leaves produced subsequent to infection. Small veins at or near the base of invaded leaves become chlorotic or necrotic. Often cells

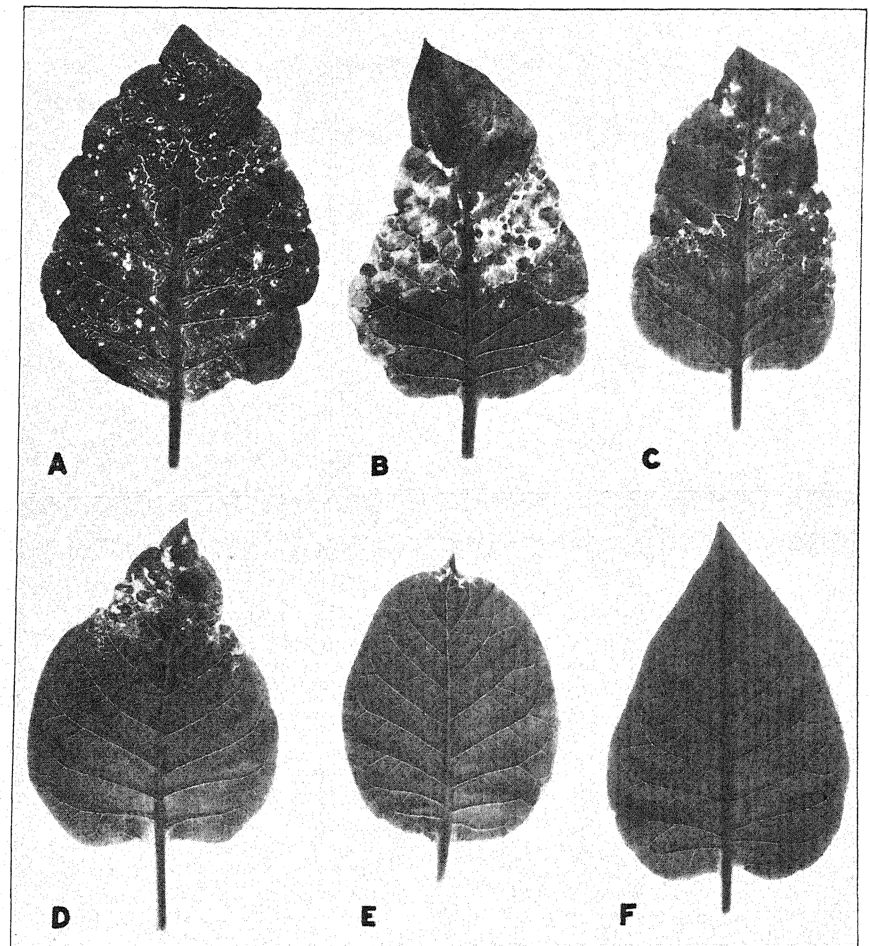

FIGURE 2. Leaves showing successive stages of recovery of Turkish tobacco plants from ring-spot: A, leaf from a plant during an acute attack; B, C, D, and E, successively increasing areas of basal portions of leaves free from symptoms; F, healthy-appearing leaf from a recovered plant.

of the intervenal portions of such leaves are killed, resulting in the formation of large necrotic areas. These areas are sometimes limited to the half of the leaf on one side of the midvein but frequently appear on both halves.

One of the most striking features of ring-spot is that plants of all the species of *Nicotiana* tested eventually recovered from the disease. The first signs of recovery are wavy lines of chlorotic or necrotic tissue extending across tip portions of young leaves and the absence of necrosis or chlorosis on basal portions of such leaves (Fig. 2 B). The wavy lines usually extend horizontally or diagonally across the leaf but occasionally follow veins and

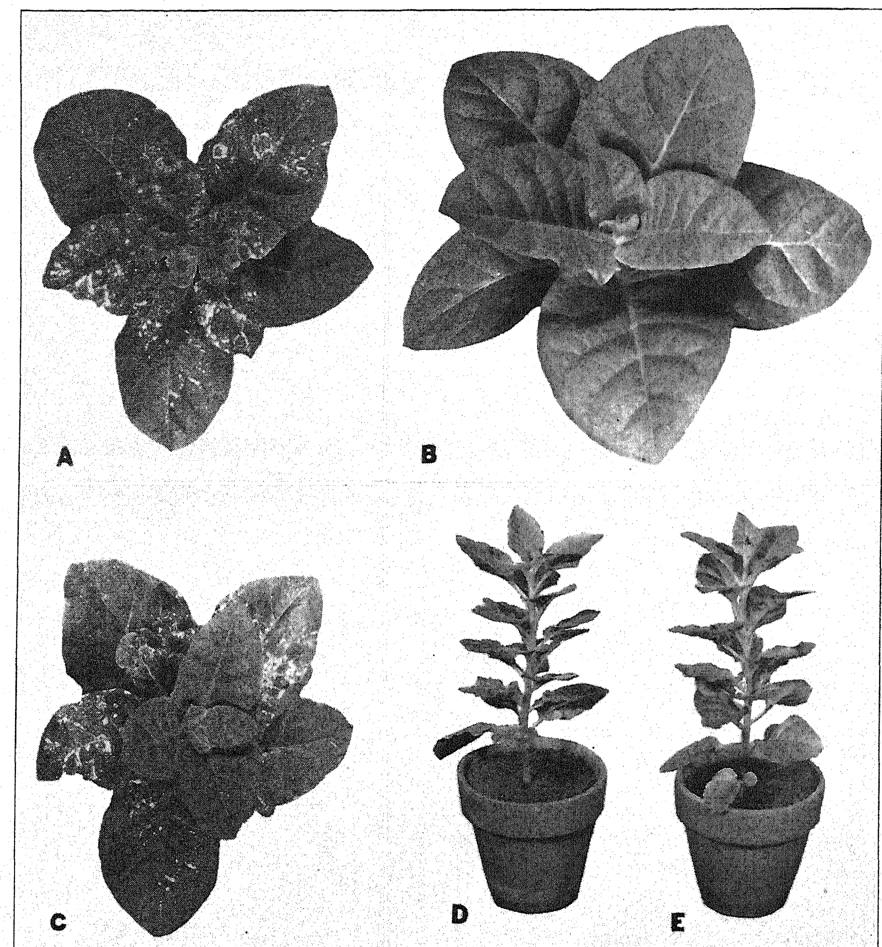

FIGURE 3. Recovery of Turkish tobacco from ring-spot: A, plant suffering from an acute attack of the disease; B, healthy control of the same age; C, same plant as A after producing several healthy-appearing leaves; D, recovered plant with many healthy-appearing leaves; E, healthy control.

produce oak-leaf patterns (Fig. 2 C) as described by Fromme, Wingard, and Priode (15). On the first leaf to develop necrotic or chlorotic lines, the lines are located near the base of the leaf and zonate lesions occur on the tip portions. As new leaves are formed, the lines occur closer and closer to the apical portions of the leaves (Fig. 2 D and E). Finally, leaves are produced which are free from chlorotic and necrotic lesions and can not be distinguished from healthy leaves (Fig. 2 F). Leaves produced after this time have never been observed to develop lesions characteristic of the onset of the disease.

The course of the disease in Turkish tobacco is illustrated by the photographs presented in Figure 3. The plant shown in A exhibits lesions characteristic of the systemic phase of the disease. Considerable leaf distortion often appears at this stage. The photograph B was taken at the same scale

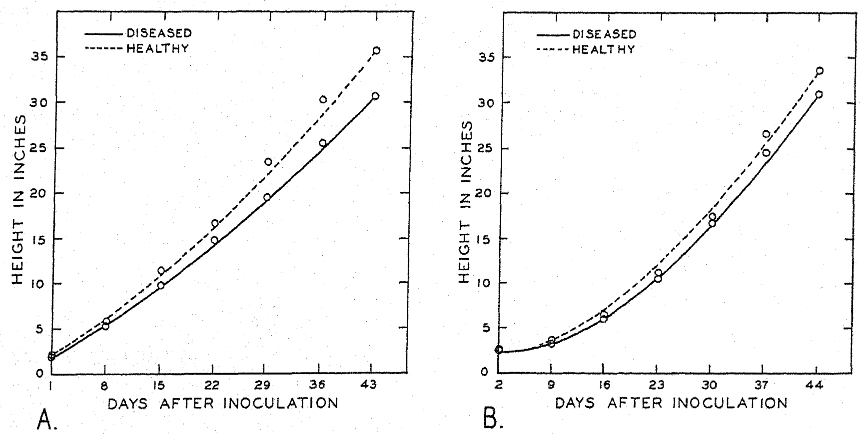

FIGURE 4. Growth rate of Turkish tobacco plants infected with ring-spot virus compared with the growth rate of healthy plants: A, plants grown in eight-inch pots in a greenhouse; B, plants grown under field conditions.

as A and shows a healthy plant of the same age for comparison. The plant shown in C has begun to recover and has produced several healthy-appearing leaves. D shows a fully recovered plant which could not be distinguished from the healthy control (E) except by the lesions on old leaves near the base.

Although recovered plants do not show chlorotic or necrotic spots, they appear to grow somewhat less rapidly than healthy plants. The difference in rate of growth of recovered and healthy plants is illustrated in the curves presented in Figure 4. The curve shown in Figure 4 A illustrates the growth rate of 19 Turkish tobacco plants infected with ring-spot virus and grown in six-inch pots in a greenhouse, as determined by the average heights of the plants measured at weekly intervals. A curve showing the growth rate

of 19 healthy plants, under the same conditions, is presented for comparison. A similar curve representing the growth rate of 70 Turkish tobacco plants infected with ring-spot virus and grown under field conditions in the summer of 1931 is shown in Figure 4 B. The other curve in this figure shows the growth rate of healthy controls. The curves show that the diseased plants grew more slowly than the healthy plants. How much of the stunting was the result of the initial shock of the disease has not been determined.

It has been observed that recovered plants occasionally show symptoms of disease. These symptoms may consist of slightly chlorotic or necrotic leaf margins and browned, distorted leaf tips. Tissues adjacent to the midvein may also become chlorotic. The leaves of recovered plants often appear slightly darker in color and have a somewhat more leathery texture than those of healthy plants. Zonate lesions characteristic of ring-spot have never been observed on leaves produced after recovery.

Effect of environmental conditions on symptoms. The difference in appearance of the ring-spot disease on plants grown in a greenhouse at different times suggested that symptoms of this disease were influenced by the conditions under which the infected plants were grown. Experiments were therefore conducted to determine whether the symptoms of ring-spot could be altered by changing the environmental conditions. The object of these experiments was not to determine the influence of any particular factor but to determine whether similar symptoms were produced under a a wide range of conditions. Also it was thought desirable to determine whether recovery from the disease occurred under this wide range of conditions.

In order to test the effect of high humidity on the production of symptoms, diseased plants were grown under large bell jars. Five young Turkish tobacco plants were rubbed with juice from ring-spot plants and immediately placed under jars which were kept on a greenhouse bench. Controls consisted of similar plants inoculated and kept uncovered in the same house. Local lesions did not appear on the plants under the bell jars until the sixth day after inoculation although they developed on the controls in three days. As the lesions increased in size, striking differences were observed. Plants in the humid atmosphere developed spots consisting of as many as eight concentric rings of necrotic tissue and rarely of less than six concentric rings. The plants kept uncovered on the same bench developed lesons with only two, three, or four rings. Similar differences were observed in the systemic lesions produced on these plants. Plants under the bell jars developed systemic lesions only after 11 days whereas the plants not under bell jars showed systemic lesions in six days after inoculation. Control plants showed more leaf distortion than plants grown under bell jars.

In the experiment just described, the light intensity was lower under

the bell jars than outside, but the temperature, as well as the humidity, was higher. A similar experiment was conducted in which controls were kept under the same light and temperature conditions but not under the same humidity conditions. Five test plants were grown under bell jars as in the previous experiment. Five control plants were grown under bell jars similar to those used for the test plants but the air in these jars was kept dry by exposure to calcium chloride. Five other plants were grown under normal greenhouse conditions. As might be expected, none of the plants under the bell jars grew normally. Those in the humid atmosphere were succulent and spindly while the plants in the dry atmosphere were considerably stunted. All 15 plants were inoculated with ring-spot virus. The symptoms on the plants grown in a humid atmosphere under bell jars were similar to those produced under the same conditions in the previous experiment. The usual ring-spot symptoms developed on the plants grown under normal greenhouse conditions. Plants grown in a dry atmosphere produced spots consisting of more delicate and more uniform necrotic rings than those occurring on plants grown under the usual greenhouse conditions. These spots showed fewer rings of necrotic tissue than those developed on plants grown in a humid atmosphere. Fewer systemic infection centers appeared on the plants in the dry atmosphere than on those in the humid atmosphere. All the plants in this experiment recovered from ring-spot and produced leaves free of necrotic or chlorotic spots. These results show that, although the symptoms of Turkish tobacco plants infected with ring-spot and grown under different conditions were not identical, the plants eventually recovered from the disease under all the conditions tested.

An experiment was undertaken to determine the effect of darkness on ring-spot symptoms. Fourteen young Turkish tobacco plants were inoculated with ring-spot. Two of these plants were placed under bell jars as in previous experiments. Two of the plants were placed under bell jars which were covered with a black cloth to exclude light. Five were placed in a dark cabinet in a laboratory. The remaining five plants were placed on a laboratory table. Local and systemic lesions appeared on all the inoculated plants but their development was considerably delayed on the plants grown under the uncovered bell jars. The symptoms on the latter were similar to those described previously on plants grown under similar conditions. Local lesions on plants kept in the dark appeared as circular water-soaked areas which increased in size and eventually involved and killed the entire leaf on which they occurred. The young tip leaves and the stems of these plants were invaded and killed at a later date. Uninoculated plants kept in the dark cabinet turned yellow but did not develop lesions and they grew normally when returned to the light.

In order to obtain more information regarding the effect of light and

humidity on ring-spot symptoms, a more comprehensive experiment was undertaken. Young Turkish tobacco plants were inoculated with ring-spot and kept for 20 days under the following conditions; two plants under bell jars in a greenhouse, two plants under bell jars which were covered with

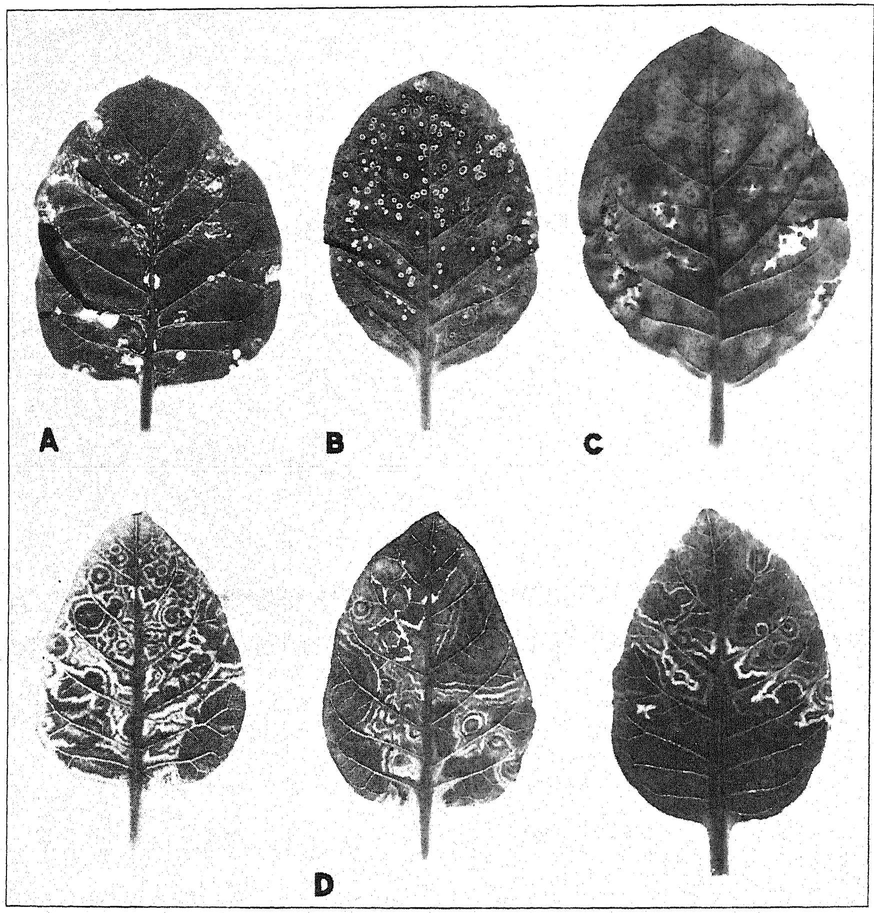

FIGURE 5. Effect of environmental conditions on symptoms of ring-spot: A, a typical leaf from a plant grown under normal greenhouse conditions; B, a typical leaf from a plant grown in deep shade; C, solid necrotic spots on a typical leaf from a plant grown in darkness; D, three typical leaves, from plants grown under a bell jar in a greenhouse, showing a large number of yellow rings. The leaf to the extreme right in D was taken when recovery was beginning and shows the necrosis confined to the apical half of the leaf.

black cloth, two plants under bell jars which were covered with black cloth from 10 a.m. to 2 p.m. each day, five plants on a greenhouse bench, five plants on the same bench but shaded, five plants shaded from 10 a.m.

to 2 p.m. each day, five plants in a dark cabinet in a laboratory, five plants near a window in the same laboratory, and five plants under constant illumination from a 500 watt lamp. The results of this experiment were similar to those obtained previously. In the absence of light, infected plants showed lesions consisting of water-soaked areas or solid necrotic spots (Fig. 5 C). These appeared on inoculated leaves, on young tip leaves, on stems, and finally caused the death of plants on which they occurred. Similar lesions were not produced on uninoculated plants grown under the same conditions. Plants kept shaded all the time or for four hours each day produced large numbers of lesions consisting of fine necrotic rings separated by wider rings of green tissue (Fig. 5 B). These lesions were similar to but distinct from those produced on the plants kept under bell jars (Fig. 5 D). The lesions which appeared on the plants kept in constant light were few in number but similar to those produced under normal conditions in the greenhouse (Fig. 5 A).

In order to determine the effect of quality of light on ring-spot symptoms, plants were grown in greenhouses kept at the same temperature and humidity but transmitting light of different wave lengths. Table I shows

TABLE I

TRANSMISSION OF FILTERS USED IN SPECTRAL HOUSES

House No.	Wave lengths transmitted (millimicrons)	Per cent solar energy transmitted
1	312–720	64
2	290–720	77
3	374–585	11
4	472–520	43
5	529–720	35

the wave lengths and per cent of solar energy transmitted by the glass of these houses. It should be stated here that although House No. 5 transmits 35 per cent of the solar energy, much of this is in the infra-red region and the intensity of visible light is therefore low in this house. Eight Turkish tobacco plants were placed in each house and inoculated three days later with ring-spot virus. Symptoms which developed on plants grown in Houses No. 1, 2, and 4 were similar to the usual symptoms of ring-spot. Symptoms on plants grown in House No. 3 were similar to those described previously for plants grown in shade and illustrated in Figure 5 B. Symptoms appearing on plants grown in House No. 5 were intermediate. These results indicate that the symptoms produced were correlated with light intensity rather than with light quality since the symptoms differed from the usual ring-spot symptoms only in Houses No. 3 and 5 in which the light intensity was low. It may be that light quality also has an

effect on symptoms of ring-spot but if so the effect was not detected in this experiment.

It may be concluded from the results of these experiments that the symptoms of ring-spot vary with the environmental conditions under which the plants are grown. It is suggested, therefore, that in order to compare symptoms produced by virus samples from different sources, the environmental conditions should be carefully controlled.

It should be pointed out in this connection that, although the symptoms produced under the conditions reported above were strikingly different, Turkish tobacco plants recovered from ring-spot under all the conditions tested except those in which the plants were killed. Plants kept in complete darkness were killed by ring-spot. Recovery occurred in the usual time in plants grown under shade or in high humidity.

RECOVERY AND IMMUNITY IN NICOTIANA TABACUM VAR. TURKISH

Recovery of Turkish tobacco from ring-spot was observed in several preliminary experiments with this disease. More comprehensive studies were then undertaken to gain information about the nature of this recovery. These studies involved tests on several species of *Nicotiana* to determine whether the recovered plants contained virus and whether symptoms could be produced on them by reinoculation. The effect of inoculating recovered and healthy plants involved in these tests is summarized in Table II.

In a study conducted with Turkish tobacco, ring-spot was transferred to 20 young plants of this variety. Typical necrotic lesions developed on all the inoculated leaves in three days and were followed by systemic symptoms which appeared on the sixth day after inoculation. Symptoms which were present on the young leaves of some of the test plants 13 days after inoculation suggested that these plants were beginning to recover. The process of recovery continued until, on the 18th day after inoculation, all the plants in the set had produced leaves on which necrotic or chlorotic symptoms did not appear and which could not be distinguished from leaves of healthy plants. Typical symptoms of ring-spot did not reappear on the recovered plants although they were grown to maturity. In order to determine whether virus was present in the recovered plants, inoculation tests were conducted 35 days after the plants had been infected. Juice from an apparently normal leaf of each of the 20 recovered plants was rubbed over the leaves of three healthy Turkish tobacco plants. All 60 test plants inoculated with juice from recovered plants developed typical ring-spot symptoms. Uninoculated controls remained healthy. These results showed that ring-spot virus was present in each recovered plant tested.

The 20 plants which had recovered from ring-spot were then tested to

determine whether symptoms could be produced on them by reinoculation. Each plant in the experiment was inoculated with ring-spot virus. Symptoms did not appear on any of these plants after inoculation. Ten healthy plants inoculated with the same virus sample developed ring-spot symptoms.

TABLE II

EFFECT OF INOCULATING RECOVERED AND HEALTHY PLANTS WITH RING-SPOT VIRUS

No. plants inoculated	Kind of plants	Previous condition	No. plants developing ring-spot lesions
123	*N. tabacum* var. Turkish	Recovered	0
95	" " " "	Healthy	95
21	" " " Burley	Recovered	0
21	" " " "	Healthy	21
19	" " " *auriculata*	Recovered	0
19	" " " "	Healthy	19
11	" " " *purpurea*	Recovered	0
11	" " " "	Healthy	11
18	" " " *angustifolia*	Recovered	0
18	" " " "	Healthy	18
9	" " " *calycina*	Recovered	0
9	" " " "	Healthy	8
10	" " " *colossea*	Recovered	0
10	" " " "	Healthy	10
3	" " " *gigantea*	Recovered	0
3	" " " "	Healthy	3
10	" " " *macrophylla*	Recovered	0
10	" " " "	Healthy	10
5	" " " Little Orinoca	Recovered	0
5	" " " " "	Healthy	5
33	" *langsdorffi*	Recovered	0
32	" "	Healthy	24
31	" *sylvestris*	Recovered	0
31	" "	Healthy	31
10	" *quadrivalvis*	Recovered	0
10	" "	Healthy	10
9	" " var. *multivalvis*	Recovered	0
9	" " " "	Healthy	9

In a similar experiment, ring-spot was transferred to eight young Turkish tobacco plants by means of the rubbing method of inoculation. Leaves of four similar plants, used as controls, were rubbed with a cheesecloth pad saturated with water. All the inoculated plants developed ring-spot symptoms in the usual time while all the controls remained healthy. In 14 days after inoculation, the diseased plants had begun to produce leaves which were free of necrotic symptoms. All the leaves produced by the recovered plants after this time appeared normal and could not be distinguished from leaves of the controls. On the 28th day after inoculation, leaves of the recovered plants and a similar number of leaves of the controls were inoculated by rubbing them with ring-spot extract. The control plants

developed both local and systemic lesions but lesions did not occur either on the inoculated leaves or on other leaves of the recovered plants produced during the remainder of their lives. The results of these experiments show that Turkish tobacco plants recover from the ring-spot disease, under the conditions of the experiments, and produce leaves on which the necrotic symptoms of the disease do not develop even when they are inoculated with ring-spot virus.

In the experiment just described, although the control plants became infected with ring-spot, they did not develop severe symptoms of the disease. The writer has observed in other cases that inoculation of moderately old tobacco plants, although usually resulting in infection, is not always followed by the production of severe symptoms. It has also been observed frequently that severe symptoms appear on rapidly growing plants whereas milder symptoms appear on slowly growing plants. In view of these observations, it was thought desirable in infection studies to use plants which were in a stage of rapid growth. Such plants were obtained by growing cuttings from plants which had recovered from ring-spot. Plants used for controls were obtained from cuttings of healthy plants of the same age and were grown under the same conditions.

Cuttings were grown from 26 Turkish tobacco plants which had recovered from ring-spot and from 22 healthy plants of the same age. Individual plants in the two sets of cuttings could not be distinguished and it was only when one group was compared with the other that noticeable differences were observed. The healthy plants appeared more succulent and were slightly larger on the average than were the recovered plants. The latter were characterized by rather thick, dark green leaves. All the plants in the experiment were inoculated with juice extracted from ring-spot plants. No lesions developed on any of the recovered plants after inoculation (Fig. 6 A). The usual symptoms of ring-spot developed on all the plants grown from cuttings of plants which had never had the disease (Fig. 6 B). The results of this experiment show that rapidly growing plants propagated from cuttings of recovered plants do not develop symptoms when reinoculated with ring-spot virus. It should be stated here, however, that some of the recovered plants developed mild symptoms consisting of slightly chlorotic areas along the midvein and margins of the leaves. Such symptoms can be observed on some of the recovered plants shown in Figure 6 A. These symptoms, however, are not to be considered as a result of reinoculation since they may occur on recovered plants previous to reinoculation.

In order to determine whether immunity to the disease persists in recovered plants, a similar experiment was conducted. The 26 recovered plants used in the preceding experiment were allowed to grow until they were about 30 inches high. Cuttings were then grown from each plant in

the set. Controls were provided by growing cuttings from 26 healthy plants. The controls grew a little more rapidly than the recovered plants but otherwise there was no difference in appearance of plants of the two groups. All the plants in the experiment were then inoculated with ring-

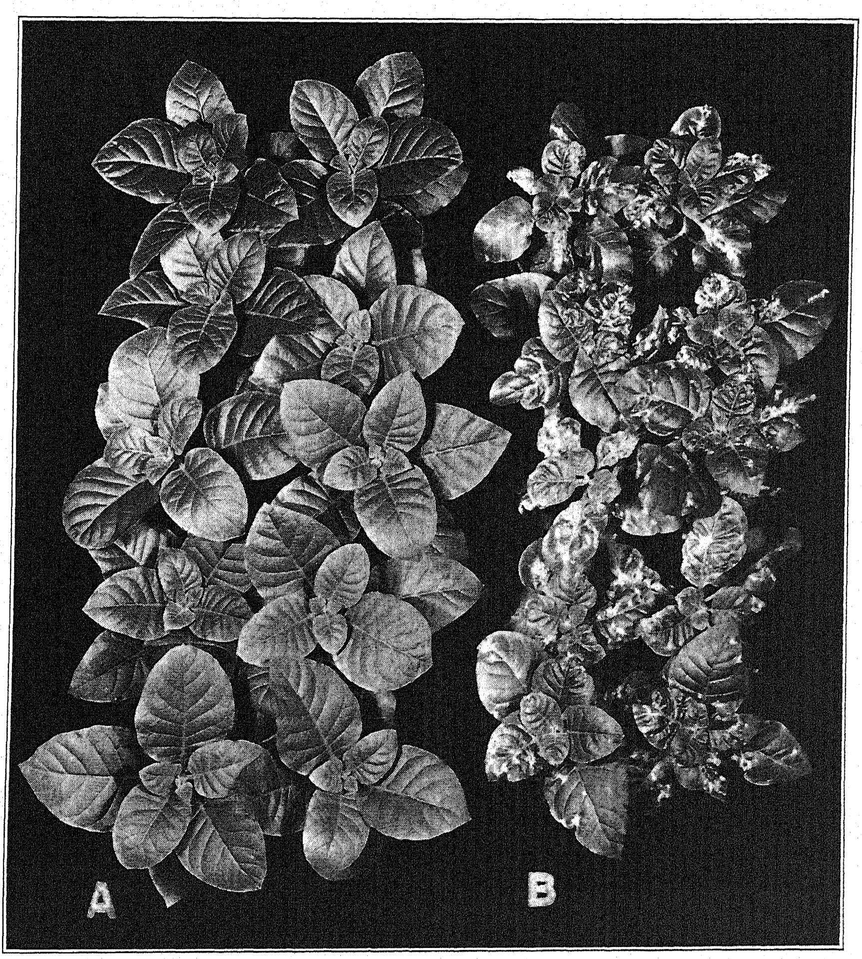

FIGURE 6. Acquired immunity in Turkish tobacco: A, plants grown from cuttings of recovered plants and inoculated with ring-spot virus; B, plants grown from cuttings of healthy plants and inoculated with the same virus sample.

spot virus by the rubbing method. The recovered plants tested did not produce lesions or other evidences of infection although all the controls developed typical symptoms of ring-spot. These results show that the re-

covered plants remained immune to the disease when propagated through two generations by cuttings.

An experiment was undertaken to determine whether this immunity would persist even longer in the recovered plants. In this experiment, all the test plants as well as the controls were obtained from a single healthy Turkish tobacco plant by vegetative propagation. In this way, the individual heritable differences were reduced to a minimum. Nine plants were grown from cuttings of a healthy Turkish tobacco plant. Five of these plants were inoculated with ring-spot and allowed to recover. The other four plants were not inoculated and served as controls. After the recovered plants had reached a height of 30 inches, seven plants were grown from them by cuttings. Similarly, six plants were grown from cuttings of the controls. All these plants were allowed to grow until they attained a height of 24 inches. Six plants were then grown from cuttings of the recovered plants and six from cuttings of the controls. These plants were allowed to grow to a height of 30 inches and cuttings were then obtained from them. Six plants were grown from cuttings of the recovered plants and six from cuttings of the controls. The young plants thus obtained were inoculated with ring-spot virus. Lesions did not develop on any of the recovered plants tested whereas lesions developed on all the healthy controls inoculated with the same virus sample.

As a further check to this experiment, plants from which the cuttings were obtained were not discarded but were allowed to produce new shoots which were then inoculated with ring-spot virus. In this way it was possible to determine whether the recovered plants in each generation of cuttings were immune or susceptible to the disease. None of the recovered plants in each of the three vegetative generations tested developed ring-spot symptoms when inoculated with the virus of this disease. All the healthy controls developed typical ring-spot symptoms when inoculated with ring-spot virus. The results of this experiment show that immunity to the ring-spot disease persisted in recovered plants grown through three vegetative generations while a similar number of healthy plants belonging to the same clon were susceptible to the disease.

As has already been pointed out in a previous section, Turkish tobacco plants which are infected with ring-spot virus and kept in the dark are killed in a short time. An experiment was conducted to determine whether recovered plants of this variety are killed when placed in the dark. A number of recovered plants grown from cuttings and a similar number of healthy controls were kept in the dark for several days. This experiment was repeated three different times. A total of 15 recovered Turkish tobacco plants were tested. All 15 of the recovered plants reacted in the same manner as did the healthy controls. Characteristic ring-spot lesions did not appear on any of the recovered plants which were kept in the dark. Some

of the recovered plants were kept in the dark for as long as eight days without being killed. These Turkish tobacco plants that had recovered from ring-spot appeared to be completely immune to the effects of ring-spot virus under the conditions of this experiment.

Effect of ring-spot virus on starch translocation. Preliminary experiments indicated that, when plants inoculated with ring-spot were kept in the dark, starch was not removed as rapidly from leaf tissue recently invaded by ring-spot virus as from surrounding tissue. It was of interest to know whether infection centers which did not appear as chlorotic or necrotic spots on leaves of Turkish tobacco could be demonstrated. An experiment was therefore conducted to determine whether such areas, if present, failed to remove starch as rapidly as healthy tissue. Leaves of several young Turkish tobacco plants were rubbed with ring-spot virus. At five o'clock on the third day after inoculation, the plants were placed in a dark room at a temperature of 10° C. The following morning they were placed in a dark cabinet at 22° C. and were left there for four to six hours. An examination of the plants at the end of this time showed that a few necrotic spots had developed on each inoculated leaf. A diagram was then made of the spots which were present on 21 of these leaves. The 21 leaves were then stained for one hour in a solution of iodine in potassium iodide (60 g. KI and 20 g. I in 3 liters of water). After this treatment the leaves displayed a large number of deeply stained spots. In most of the leaves there were more of these spots than necrotic spots as shown by comparison with the diagrams previously made, but in a few of the leaves the deeply stained spots appeared only where necrotic or chlorotic lesions were previously observed. These results show that areas of previously healthy leaves of Turkish tobacco invaded by ring-spot virus but not exhibiting necrotic or chlorotic symptoms could be detected by staining in iodine.

Since the results of previous experiments showed that inoculation of leaves of recovered plants with ring-spot virus did not lead to the production of necrotic or chlorotic symptoms, an experiment was undertaken to determine if symptoms could be demonstrated on such leaves after inoculation by staining them with iodine. The leaves studied in this experiment were stained with iodine four or seven days after inoculation. Although areas invaded by the virus were detected on previously healthy plants inoculated at the same time, similar lesions were not produced on inoculated leaves of the nine recovered plants tested. Likewise, lesions could not be detected on uninoculated leaves of six recovered plants. It was observed, however, that some of the young tip leaves of recovered plants failed to remove starch as readily as similar leaves on healthy plants. These symptoms were suggestive of the chlorotic or necrotic areas which occasionally appear on tip portions of leaves of recovered plants. The results of this experiment show that although the inoculation of leaves

of previously healthy plants led to the development of areas in which starch removal was delayed, similar areas did not develop as a result of inoculation of leaves of any of the recovered plants tested. Inoculation of recovered plants did not result in production of symptoms under the conditions of this experiment.

Symptoms produced by virus from recovered plants. As shown by previous experiments, ring-spot virus is present in recovered Turkish tobacco plants. Symptoms produced after inoculation with virus from the recovered plants appeared to be identical with those produced by virus from severely diseased plants. In an experiment conducted to determine this point more carefully, 26 young Turkish tobacco plants were inoculated with virus obtained from recovered plants and 26 similar plants were inoculated with virus from plants which showed zonate spots characteristic of the ring-spot disease. In order to compare several different virus samples, each test plant was inoculated with virus from a different source. The test plants were of the same age and were grown under the same environmental conditions. Symptoms which developed on 26 Turkish tobacco plants inoculated with virus secured from recovered plants were identical with those produced by inoculation of 26 similar plants with virus secured from plants in the acute stage of the disease. These results show that ring-spot virus was not attenuated in any of the recovered plants tested.

Transmission by grafting. In previous experiments, it was shown that virus from recovered plants is infectious when applied to leaves of healthy plants. This fact suggests that protective substances, if present in the juice from recovered plants, are not carried over in any appreciable quantity by the rubbing method of inoculation. It was thought, however, that if such substances were present they might be demonstrated by intergrafting healthy and recovered plants. In a preliminary experiment to determine whether ring-spot could be transmitted by intergrafting healthy plants with plants showing necrotic spots, three healthy Turkish tobacco plants grafted on ring-spot plants became diseased. A similar number of healthy plants grafted on healthy plants did not develop ring-spot symptoms. An experiment was conducted to determine whether a similar result could be obtained by intergrafting healthy and recovered plants. Six scions cut from healthy Turkish tobacco plants were grafted on Turkish tobacco plants grown from cuttings of recovered plants. All six of these previously healthy plants developed typical ring-spot symptoms in 15 days. Four scions from healthy Turkish tobacco plants grafted on healthy plants did not develop symptoms. Two scions cut from recovered plants were grafted to healthy plants. Shoots which grew from the healthy plants below the graft union developed typical ring-spot symptoms but lesions did not appear on the recovered portions of the grafted plants. Two recovered plants intergrafted with severely diseased plants did not develop

ring-spot symptoms. The results of this experiment show that ring-spot is transmitted by intergrafting healthy and recovered plants under the conditions of the experiment. This disease is transmitted from recovered plants when healthy plants are used for stocks or when they are used for scions. If protective substances were present in Turkish tobacco plants infected with ring-spot, they were not detected in this experiment.

Movement of virus in ring-spot plants. In order to determine the approximate rate of movement of virus in Turkish tobacco plants, an experiment was conducted in which the relative virus concentrations of different parts of infected plants were measured at intervals after inoculation. In this experiment five-inch Turkish tobacco plants were inoculated by rubbing juice from ring-spot plants over a single leaf of each plant. At definite intervals after inoculation, an infected plant was divided into sections which were then tested for virus concentration. The concentration of virus in the inoculated leaf, in the leaf immediately above the inoculated leaf, in the leaf immediately below, in a three-inch portion of the stem above the inoculated leaf, in a similar stem section below the point of inoculation, in the tip leaves, and in the roots was determined. The concentration of virus in a leaf bearing necrotic spots was also determined after such leaves appeared. Each determination was made by inoculation of 16 leaves of Black cowpeas. The numbers of lesions which developed on the inoculated leaves showed the relative concentration of virus in the inoculum. The results of this experiment are presented in Table III.

TABLE III

NUMBER OF LESIONS APPEARING ON 16 LEAVES OF BLACK COWPEA INOCULATED WITH RING-SPOT VIRUS FROM PORTIONS OF TURKISH TOBACCO PLANTS

Days after inoculation of tobacco plants	Portion of tobacco plants used as inoculum							
	Inoculated leaf	1st leaf above	1st leaf below	Stem portion above	Stem portion below	Tip leaves	Roots	First necrotic leaf below tip
1	0	0	0	0	0	0	0	—
2	10	0	0	0	0	0	0	—
3	298	0	0	0	0	0	0	—
5	2256	0	5	0	0	1	8	—
7	2001	0	0	325	0	58	4	—
10	2535	0	11	117	21	434	15	1964
21	541	0	0	136	290	724	13	1250
28	78	0	0	91	353	444	99	875
35	—	—	—	144	373	957	361	20
110	—	—	—	36	32	339	40	—

The symptoms on plants from which these virus samples were obtained were as follows: local lesions appeared on the inoculated leaves in three days, systemic lesions appeared in six days, and healthy-appearing

green leaves were produced 14 days after inoculation. On the 21st day after inoculation, many of the lower leaves, including some of the leaves tested, were dead and 35 days after inoculation all the leaves exhibiting lesions were dead.

The experiment shows that the virus reached a high concentration in the inoculated leaf before reaching a measurable concentration in other portions of the same plant and that it reached a maximum concentration in the inoculated leaf between the fifth and the tenth day after inoculation. Virus seems to have appeared simultaneously in the roots, stem, and tip of the inoculated plants. After passing to the tips of the plants, the virus did not reach a maximum concentration until after necrotic symptoms appeared. There was no significant decrease in virus concentration in the recovered portions of the plants although the concentration decreased appreciably in leaves that dried out. Finally, virus could not be detected in quantity in the older uninoculated leaves of the plants.

Since the results of the above experiment did not show significant differences in virus concentration in juice from recovered and from severely diseased plants, more extensive measurements were undertaken. Virus for these measurements was obtained from six Turkish tobacco plants grown from cuttings of recovered plants. As controls, virus was secured from six Turkish tobacco plants grown from cuttings of healthy plants and inoculated with ring-spot virus eight days previously. All the control plants which were inoculated eight days previously showed necrotic spots on the young tip leaves but necrotic or chlorotic spots were not present on any of the recovered plants tested. The virus concentration in the tip portions of these plants was determined in each case by inoculation of 16 cowpea leaves. The results of these measurements are presented in Table IV.

TABLE IV

RELATIVE VIRUS CONCENTRATIONS OF TURKISH TOBACCO PLANTS BEFORE AND AFTER RECOVERY

Plant No.	1	2	3	4	5	6	Average
Plants recovered from ring-spot	256*	50	88	168	298	456	218
Plants showing necrotic lesions	320	236	1460	692	540	384	604

* The figures represent the numbers of lesions appearing on 16 leaves of cowpeas following inoculation with the virus sample indicated.

These results suggest that the virus concentration of plants which have recovered from ring-spot is less than that of plants showing acute symptoms. However, as may be seen from the table, some of the recovered plants appear to have had a higher virus concentration than some of the controls.

The relatively low concentration of virus in some of the plants showing systemic symptoms may be due to the fact that they had not been infected for a sufficient length of time for the disease to become thoroughly systemic in them and for the virus to reach a maximum concentration in their tip portions. However, since the method has not been thoroughly studied, definite conclusions cannot be drawn. Nevertheless, the results show that recovered plants contain a relatively large quantity of virus under the conditions of the experiment.

Experiments on seed transmission. It has already been shown (18, 33) that this ring-spot disease is not transmitted to any extent through seed of Turkish tobacco. It is of considerable interest to know whether immunity to the ring-spot disease is transmitted through seed of recovered plants. An experiment to determine this point was conducted with Turkish tobacco seedlings grown from seed of plants which had recovered from ring-spot previous to flowering. Ring-spot symptoms did not appear on 1182 young plants grown from this seed. A leaf was removed from each of 104 of these plants selected at random and used to inoculate leaves of healthy Turkish tobacco plants. All the plants inoculated with juice from these leaves remained healthy but a similar number of plants inoculated with ring-spot virus became diseased. Virus was not present in any of the seedlings grown from seed of recovered plants and used as a source of inoculum. In order to determine whether such seedlings were immune to ring-spot 825 were inoculated with ring-spot virus. Typical ring-spot symptoms developed on all these seedlings. It may therefore be concluded that neither virus nor immunity to the disease is transmitted through seed of recovered Turkish tobacco plants under the conditions of this experiment.

Length of time necessary for recovery from ring-spot. In the summer of 1931, diseased plants of four species of *Nicotiana* and of ten varieties of *N. tabacum* were grown under field conditions. Many of these plants recovered and produced healthy-appearing leaves. Some plants, however, failed to recover although grown to maturity. They developed necrotic lesions on practically every leaf. A very few plants of some varieties of *N. tabacum* recovered from ring-spot under field conditions. Almost all the infected plants of other varieties recovered. In *N. tabacum* var. *purpurea*, 20 plants were infected with ring-spot and only four plants recovered. Similar results were obtained with *N. tabacum* var. *auriculata* and *N. tabacum* var. Turkish. In order to determine the reason for this behavior an experiment was conducted with Turkish tobacco plants in a greenhouse. Ten plants of this variety were transplanted to heavily fertilized soil in eight-inch pots and inoculated by rubbing virus over some of the leaves of each plant. Local lesions appeared on inoculated leaves in three days and systemic symptoms appeared four days later. Necrotic spots developed on all the leaves produced by these plants during the next

three weeks. Four weeks after inoculation, one plant produced leaves on which such spots did not develop. Five weeks after inoculation, all the plants had produced leaves which were healthy in appearance. The controls, which were inoculated at the same time and grown in four-inch pots, produced healthy-appearing leaves two weeks after inoculation. Approximately twice as many leaves showing necrotic spots were produced on the rapidly growing plants as on the more slowly growing controls. The results of this experiment show that rapidly growing plants require a longer time than slowly growing plants to recover from ring-spot, and that such plants developed more leaves on which necrotic symptoms appeared under the conditions of the experiment. It may therefore be concluded that the time of recovery of Turkish tobacco plants from ring-spot is dependent upon the growth rate of the plants. An explanation of the behavior of ring-spot in the field is suggested by the results of this experiment. It may be that under the favorable growing conditions that prevailed outside in summer, some plants were too succulent to recover during the period they were under observation.

RECOVERY AND IMMUNITY IN OTHER VARIETIES OF NICOTIANA TABACUM

The results of preceding experiments having shown that Turkish tobacco plants normally recover from ring-spot and acquire an immunity to this disease, experiments were conducted to determine whether other varieties of *N. tabacum* also exhibit this behavior. Ring-spot was transferred to 25 plants of Burley tobacco, 22 plants of *auriculata*, 25 plants of *purpurea*, 20 plants of *angustifolia*, 10 plants of *calycina*, 10 plants of *colossea*, 3 plants of *gigantea*, 10 plants of *macrophylla*, and 10 plants of Little Orinoca. An equal number of plants of each variety were not inoculated and served as controls. All the plants inoculated with ring-spot virus developed local lesions in three or four days and systemic symptoms in six or seven days after inoculation. The characteristic zonate necrotic spots appeared on inoculated leaves of all these plants and on leaves produced subsequent to inoculation. All the plants infected with ring-spot virus recovered from the disease and produced several healthy-appearing leaves in 14 to 20 days after infection. Recovered plants of most of the varieties tested could not be distinguished in appearance from the healthy controls. A few plants of the tobacco varieties *auriculata* and Burley showed mild symptoms similar to those previously described on Turkish tobacco. Zonate spots were not observed on recovered plants of any of the varieties listed above.

Tests were conducted on some of the recovered plants in these experiments to determine whether symptoms of ring-spot could be produced on them by reinoculation with virus. The rubbing method of inoculation was

employed. Nine recovered plants of the tobacco variety *angustifolia*, five of *calycina*, five of *colossea*, and five of *macrophylla* were inoculated. An equal number of healthy plants of each variety were inoculated with the same virus sample and served as controls. Ring-spot symptoms did not develop on any of the recovered plants. Typical ring-spot lesions developed on all the previously healthy plants with the exception of one plant of the variety *calycina*. Additional data on the failure of recovered plants of these varieties to develop symptoms after reinoculation are given below.

An experiment was undertaken to determine whether immunity to the ring-spot disease in recovered plants of different varieties of *N. tabacum* would persist. Seventeen plants of Burley, 15 of *auriculata*, 11 of *purpurea*, 9 of *angustifolia*, 4 of *calycina*, 5 of *colossea*, 3 of *gigantea*, 5 of *macrophylla*, and 5 of Little Orinoca were grown from cuttings of recovered plants. An equal number of plants of each of these varieties were grown from cuttings of healthy plants and were used as controls. All of these plants were inoculated with ring-spot virus. Symptoms did not develop on any of the recovered plants. Ring-spot lesions were produced on all the healthy plants inoculated with the same virus sample. It is concluded that immunity to ring-spot persisted in recovered plants of all nine varieties when grown through one generation by cuttings.

Some of the plants of this experiment are shown in Figures 7 and 8. Figure 7 A shows a plant of Burley tobacco grown from a cutting of a recovered plant and reinoculated with ring-spot virus. It may be seen that this plant is free of lesions while the control plant (Fig. 7 B) inoculated at the same time is severely diseased. Figure 7 C shows a healthy-appearing plant grown from a cutting of a recovered plant of the variety *auriculata*. No lesions appear on this plant although it was inoculated with ring-spot virus at the same time as the control (Fig. 7 D). The plant shown in Figure 7 E was grown from a cutting of a recovered plant of *purpurea* and was subsequently reinoculated with ring-spot virus. It can be seen that this plant appears healthy whereas the control (Fig. 7 F) displays characteristic ring-spot symptoms. One of the plants grown from cuttings of recovered plants of *N. tabacum* var. Little Orinoca is shown in Figure 8 A. Ring-spot symptoms did not occur on this plant although the healthy plant inoculated with the same virus sample developed characteristic necrotic spots (Fig. 8 B).

Since it was shown in previous experiments that virus is invariably present in plants of Turkish tobacco which have recovered from ring-spot, some of the recovered plants of the other varieties (of *N. tabacum*) reported above were tested to determine whether they contained ring-spot virus. This test was conducted with young plants grown from cuttings of recovered plants but not reinoculated. In each case, the test for presence of virus was made by inoculating two to six leaves of Black cowpeas with

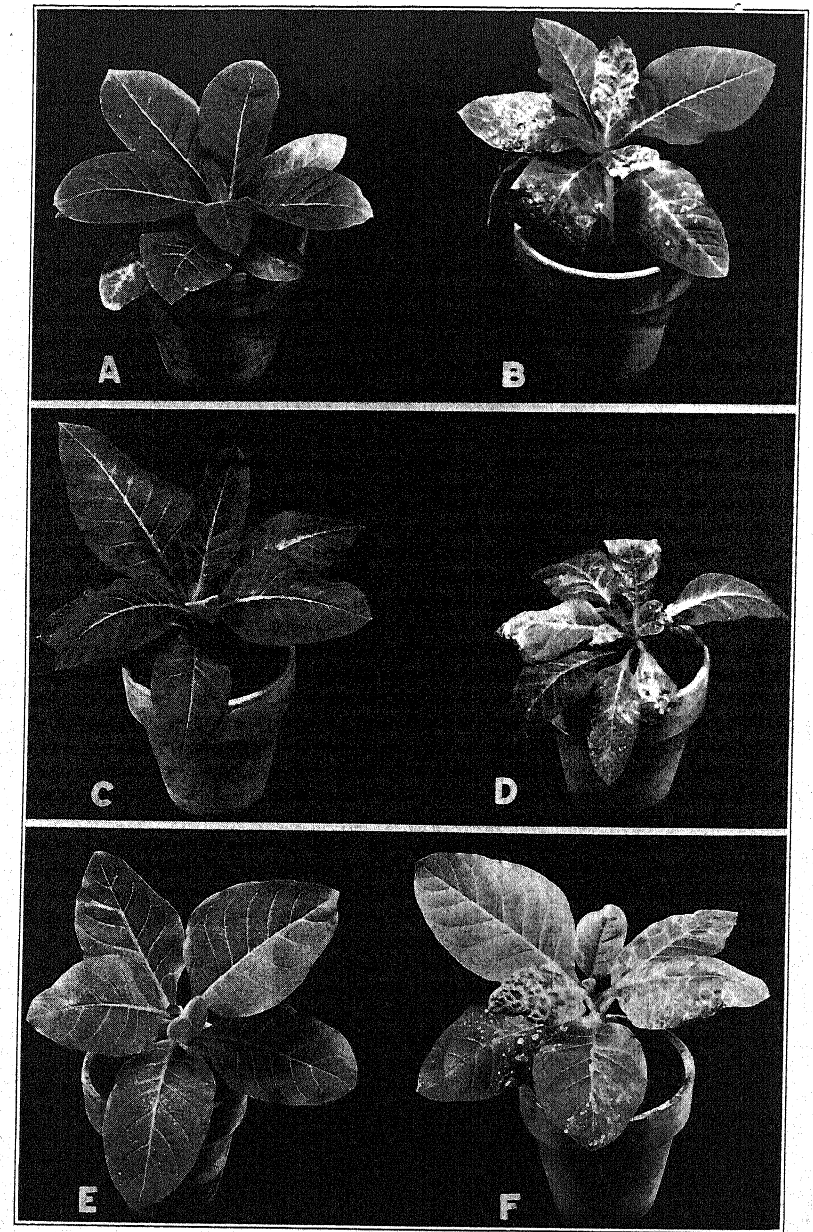

FIGURE 7. Effect of inoculating plants grown from cuttings of recovered and of healthy plants: *N. tabacum* var. Burley, A, recovered plant showing no necrotic spots, B, previously healthy control showing typical necrotic spots; *N. tabacum* var. *auriculata*, C, recovered plant, D, control; *N. tabacum* var. *purpurea*, E, recovered plant, F, control.

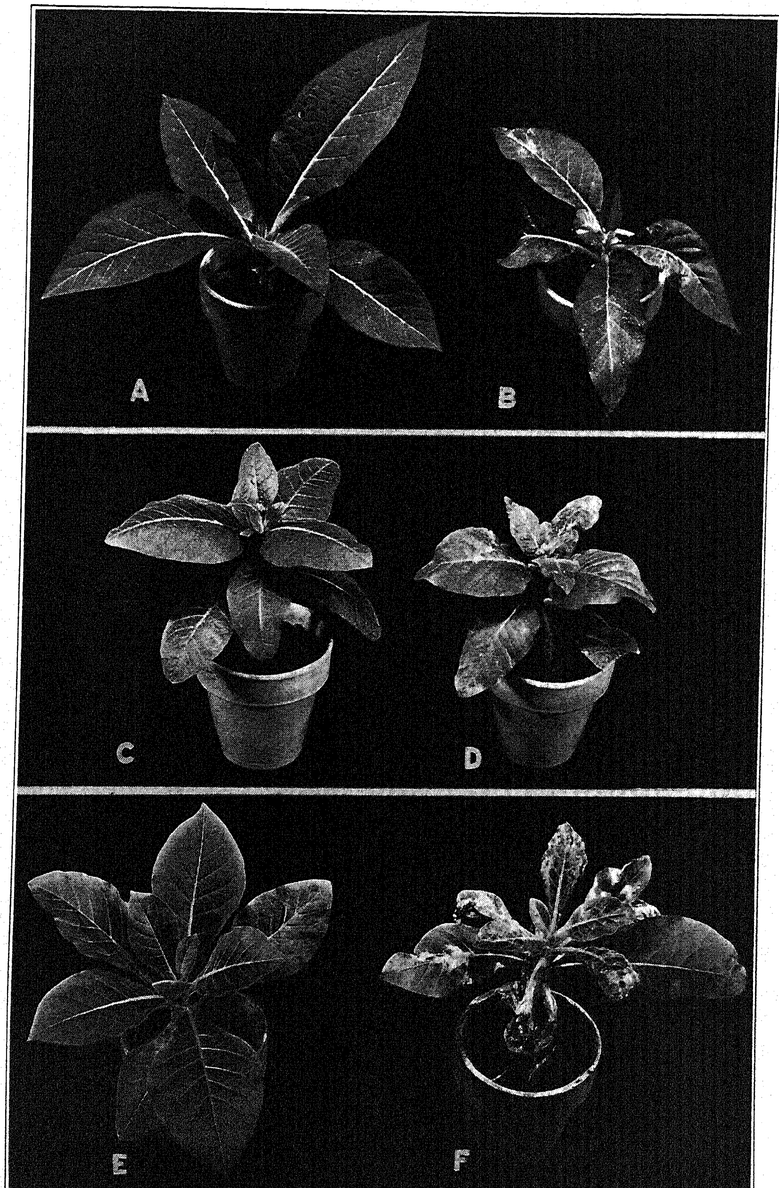

FIGURE 8. Effect of inoculating plants grown from cuttings of recovered and of healthy plants: *N. tabacum* var. Little Orinoca, A, recovered plant showing no necrotic spots, B, previously healthy control showing typical necrotic spots; *N. langsdorffi*, C, recovered plant, D, control; *N. sylvestris*, E, recovered plant, F, control.

juice obtained from a healthy-appearing leaf. Controls consisted of two to six leaves of Black cowpeas inoculated with juice from leaves of an equal number of healthy plants of each of these varieties. Nine recovered plants of the variety *angustifolia*, five of *calycina*, four of *colossea*, five of *macrophylla*, and five of Little Orinoca were tested for presence of virus as outlined above. Cowpea leaves inoculated with juice from each of these recovered plants developed typical ring-spot lesions but such lesions did not develop on any of the cowpea leaves inoculated with juice from the healthy controls. These results show that virus was present in each recovered plant tested.

It may be concluded from the results of the experiments reported above that plants of the nine varieties tested acquire a lasting immunity to the ring-spot disease under the conditions of these experiments.

RECOVERY AND IMMUNITY IN OTHER SPECIES OF NICOTIANA

Nicotiana langsdorffi Schrank. Recovery was observed in 25 plants of *N. langsdorffi* previously inoculated with ring-spot virus. Reinoculation of these recovered plants did not lead to the production of ring-spot symptoms during the month the plants were under observation. Of 25 healthy control plants inoculated with the same virus sample, 17 developed ring-spot symptoms. A similar experiment was conducted with eight plants grown from cuttings of recovered plants and seven plants grown from cuttings of healthy plants. Although reinoculated, the recovered plants did not develop lesions during the two months they were under observation (Fig. 8 C). All the controls inoculated with the same virus sample became diseased (Fig. 8 D). It is concluded that the recovered plants were immune or highly resistant to the effects of the ring-spot virus under the conditions of this experiment.

Nicotiana sylvestris Spegaz. & Comes. Twenty-five plants of *N. sylvestris* were observed to recover from ring-spot. Plants were grown from cuttings of all the recovered plants and from 25 healthy plants of the same age. A test for presence of virus was made by inoculation of a healthy Turkish tobacco plant with juice extracted from each recovered plant. All the controls were tested for virus in the same manner. The 25 Turkish tobacco plants inoculated with juice from the recovered plants developed typical ring-spot symptoms whereas such symptoms did not appear on 25 Turkish tobacco plants inoculated with juice from the healthy controls. These results show that ring-spot virus was present in all the recovered plants tested. All 25 recovered plants were reinoculated with ring-spot virus. Symptoms did not develop on any of the recovered plants in the three months they were under observation, although all the controls inoculated with the same virus sample developed typical ring-spot

symptoms. It is concluded from these results that the recovered plants of *N. sylvestris* which were tested acquired an immunity to ring-spot.

In order to determine whether this immunity persists in plants of *N. sylvestris*, six plants grown from cuttings of recovered plants were inoculated with ring-spot virus. Controls were provided by inoculation of six plants grown from cuttings of healthy plants. As in the previous experiment, symptoms did not develop on these recovered plants but all the controls inoculated with the same virus sample developed typical ring-spot symptoms. One of the recovered plants from this experiment is shown in Figure 8 E. This plant appeared healthy. The control (Fig. 8 F), inoculated at the same time, was badly diseased. The recovered plants were kept under observation for 140 days but ring-spot symptoms did not develop on them at any time during this period. To determine whether immunity to ring-spot would persist even longer in these recovered plants, six cuttings were grown from them and, when of a suitable size, were reinoculated. They did not develop symptoms of ring-spot although six healthy Turkish tobacco plants inoculated with the same virus sample became diseased. From these results it is concluded that the *N. sylvestris* plants which were infected with ring-spot recovered from the disease and produced leaves which, although containing virus, were free from chlorotic and necrotic symptoms and did not develop such symptoms even on reinoculation. It should be stated here that the recovered plants were kept under observation for more than four months but did not develop any symptoms such as were occasionally observed on recovered plants of some varieties of *N. tabacum*. The recovered plants, although containing virus, could not be distinguished from healthy plants except by inoculation tests.

Nicotiana quadrivalvis Pursh. and Nicotiana quadrivalvis var. multivalvis Gray. Recovery was observed in ten *N. quadrivalvis* plants and in nine *N. quadrivalvis* var. *multivalvis* plants. Virus was shown to be present in a healthy-appearing leaf of each recovered plant by inoculation of two cowpea plants with juice extracted from such leaves. Controls, which consisted of cowpea plants inoculated with juice from an equal number of healthy plants, did not develop lesions. The 19 recovered plants were reinoculated but did not develop symptoms although symptoms appeared on 19 healthy plants inoculated with the same virus sample. These results show that plants of *N. quadrivalvis* and of *N. quadrivalvis* var. *multivalvis* recover from ring-spot and acquire an immunity to the disease under the conditions of the experiment.

IMMUNITY WITHOUT AN ATTACK OF THE DISEASE

The results of a previous experiment showed that ring-spot virus reached a high concentration at the point of inoculation before moving to

other parts of the plant. It was also observed that the infected plants began to recover at or shortly after the time when virus could be detected in the young tip leaves. This observation suggested that plants might acquire immunity to ring-spot without showing symptoms of the disease if virus was inoculated into tissue near the growing point. Experiments were conducted in which this method of inoculation was attempted. In the first of these experiments, 12 Turkish tobacco plants were inoculated by puncturing apical portions of their stems with needles previously dipped in juice from ring-spot plants. Symptoms appeared on three of these plants after 11 days. The other nine plants remained apparently healthy for 24 days and the tops of these healthy-appearing plants were cut off with a sterile scalpel. Typical ring-spot symptoms appeared on shoots which grew on six of these plants. Symptoms did not appear on the other three plants. These results indicate that although virus was present in the stems of some of the plants, it did not move into the young leaves until after the plants were cut back.

In a similar experiment, buds from ring-spot plants were inserted into the stems of 11 Turkish tobacco plants when they were about eight inches high. Ring-spot symptoms did not appear on any of the budded plants in 15 days, one plant showed symptoms on the 17th day, one on the 18th day, and one on the 22nd day after budding. The other eight plants were cut back with a sterile scalpel. Four of the plants were cut back on the 15th day after inoculation, three on the 28th day, and one on the 40th day after inoculation. Although none of the budded plants showed symptoms at the time they were cut back, the new shoots that developed on all eight plants showed typical ring-spot symptoms. All the plants in the experiment recovered in 10 to 14 days after symptoms first appeared. As a control to this experiment, a similar number of recovered plants were cut back with a sterile scalpel. None of the shoots produced by these plants developed ring-spot symptoms although inoculation tests showed that they contained ring-spot virus. It can be concluded, therefore, that the budded plants contained ring-spot virus but that this virus did not move to the growing points until after the plants were cut back.

A similar experiment was conducted, using different methods of inoculations. Eight Turkish tobacco plants about 12 inches high were inoculated by rubbing ring-spot virus over the upper surfaces of two of the old leaves of each plant. Eight similar plants were inoculated by puncturing the stems near the base with pins dipped in virulent juice. Eight similar plants were inoculated by inserting diseased buds into their stems. The points of inoculation in each case were between three and five inches above the ground level. Ring-spot symptoms did not appear on any of the inoculated plants after 19 days when they were cut back with a sterile scalpel. Lesions developed on the new shoots produced by five of the budded plants

and by seven of the plants inoculated with pin punctures. Symptoms did not develop on the other 12 plants. Whether the plants that did not show symptoms were infected was not determined.

It may be concluded from the results of these experiments that although ring-spot virus may be present in certain portions of Turkish tobacco plants for a considerable length of time, other portions of such plants may remain susceptible to attack.

The methods previously employed having failed to introduce appreciable quantities of virus directly into the growing point, other methods were attempted. In one such experiment, four young Turkish tobacco plants were inoculated by rubbing virus over a single leaf. All the leaves above the inoculated one were removed. The leaves which developed subsequently on these plants were removed as soon as they were large enough to handle without injury to the young buds. No ring-spot lesions were observed on any of these small leaves. After 13 days, new leaves were allowed to develop on the inoculated plants. These leaves appeared normal and did not exhibit lesions at any time, although they were shown to contain virus by inoculation of healthy plants with juice extracted from them.

In the experiment just reported, the only lesions which developed on the plants were those which formed on the inoculated leaves. A similar experiment was conducted in which the plants were inoculated by pricking virus into the stems near the young tip buds. The tip leaves were removed from six healthy Turkish tobacco plants approximately eight inches in height and the stems were inoculated with pins previously dipped into juice from diseased plants. New leaves which were formed in the next ten days were removed before they attained a length of one centimeter. After ten days the young leaves were allowed to grow from the apical bud. On four of the plants these leaves appeared healthy, and on the other two plants all but two leaves appeared healthy. The four leaves which did show symptoms developed wavy lines which were confined to the tip portions of the leaves and were typical of symptoms appearing on partially recovered plants. In order to determine whether virus was present in the apparently normal leaves, a single leaf was removed from each plant and used as a source of inoculum for healthy tobacco plants. Typical lesions appeared on all the test plants. This result showed that virus was present in each leaf tested. In order to determine whether lesions could be produced on these plants, ring-spot virus was rubbed over the healthy-appearing leaves of each plant. The inoculated leaves did not develop ring-spot lesions although two healthy plants inoculated with the same virus sample became diseased. Although ring-spot lesions did not appear on the leaves of four inoculated plants, virus was present in the young tip leaves of these plants, and the plants developed an immunity or a high degree of resistance to the effects of the virus under the conditions of the experiment.

A similar experiment was undertaken with the tobacco varieties Burley and *auriculata*. After the removal of the tip leaves, virus was introduced into four plants of each of these varieties by puncturing their stems with needles previously dipped into infectious juice. All the new leaves which were produced during the next ten days were removed before they exceeded one centimeter in length. Symptoms were not observed on any of the leaves which were removed. After the tenth day, new leaves were allowed to grow on the four plants of each variety. Leaves which were produced on one plant of each variety showed symptoms consisting of wavy lines of necrotic tissue and typical of symptoms produced on plants recovering from ring-spot. Only healthy-appearing leaves developed on the other six plants. Tests were made to determine whether ring-spot virus was present in such leaves. Healthy Turkish tobacco plants were inoculated with juice from each plant in the experiment. All the inoculated plants developed typical ring-spot symptoms but similar plants inoculated with juice from healthy plants did not develop symptoms. This result showed that virus was present in healthy-appearing leaves of each of the inoculated plants. The healthy-appearing leaves of each plant were inoculated with ring-spot virus. Symptoms did not develop on any of the leaves although healthy plants inoculated with the same virus sample developed typical symptoms.

It may be concluded from these results that, under the conditions of the experiments, plants of the three varieties of *N. tabacum* that were tested acquired immunity or a high degree of resistance to the effects of ring-spot virus without showing symptoms of the disease.

In the experiment described above, two of the test plants developed symptoms similar to those which appear on plants during recovery from ring-spot. This result suggested that there is a definite time factor involved in recovery. A test was made to gain more information regarding this possibility. The leaves at the tops of 11 Turkish tobacco plants which were about 15 inches high were removed and virus was pricked into the apical portion of the stem of each plant. The leaves developing subsequently were removed while still very small. Later, new leaves were allowed to grow as follows: on one plant after two days, on two plants after four days, on two plants after six days, on two plants after eight days, on two plants after ten days, and on two plants after twelve days. Typical ring-spot lesions developed on the plants in which leaves were allowed to grow out in two or four days after inoculation. The leaves which grew out six to eight days after inoculation developed lesions and wavy lines only on their tip portions. The leaves which grew out ten or more days after inoculation appeared healthy but contained ring-spot virus as shown by inoculation tests. The results of this experiment indicate that the size or age of the leaf which is invaded by ring-spot virus determines the pattern

that is produced on the leaf and that invasion of very young leaves does not result in the production of ring-spot symptoms. These results also suggest that the time of recovery from ring-spot depends upon the time required for virus to reach the growing points of infected plants.

MOSAIC SYMPTOMS IN RECOVERED RING-SPOT PLANTS

During the course of these investigations, an occasional plant which had recovered from ring-spot became contaminated with tobacco mosaic virus. It was noticed that symptoms which appeared on such plants were different from the usual mosaic symptoms. Experiments were therefore undertaken to determine whether symptoms produced by tobacco mosaic virus in recovered ring-spot plants were different from those produced by the same virus in plants not infected with ring-spot virus. The mosaic virus used in these experiments was a strain of the common field type of tobacco mosaic usually referred to as strong mosaic. In the first of these experiments, 12 young Turkish tobacco plants were inoculated with ring-spot virus and allowed to recover from this disease. Six of these plants were then inoculated with tobacco mosaic virus. Six healthy plants of the same age were also inoculated with mosaic virus. Mosaic symptoms appeared on the inoculated plants in eight days. All the plants infected with mosaic alone developed symptoms considered typical for this disease (Fig. 9 B). The plants infected with both viruses developed milder mottling symptoms (Fig. 9 A). The leaves produced on these plants were less distorted and showed fewer yellow areas. As the plants matured, the differences became less marked but were sufficient to permit a separation of plants carrying the two viruses from those carrying the tobacco mosaic virus alone. Necrotic symptoms did not appear on the recovered ring-spot plants (Fig. 9 C).

The experiment was repeated and identical results were obtained. Turkish tobacco plants infected with both ring-spot and mosaic virus could be separated at any time from similar plants infected with only one of these viruses.

In a similar but more extensive experiment, 12 Turkish tobacco plants were inoculated with ring-spot virus, 18 were inoculated with mosaic virus, and 30 were inoculated with both of these viruses. Of the plants inoculated with both viruses, some were inoculated with ring-spot virus two weeks previous to inoculation with mosaic virus, while others were inoculated with both viruses at the same time. Six healthy plants were kept as controls.

Symptoms appeared on the inoculated plants after the usual period of time. Development of mosaic symptoms was delayed in the plants previously infected with ring-spot. The plants infected with mosaic virus alone developed the usual mosaic symptoms. All the plants infected with both viruses showed only a mild mottling and, although the mottling be-

came more severe as the plants matured, they could be easily distinguished at any time during the experiment from plants infected with mosaic only. The first symptoms which were produced on plants inoculated simultaneously with both viruses were much like the symptoms of strong mosaic

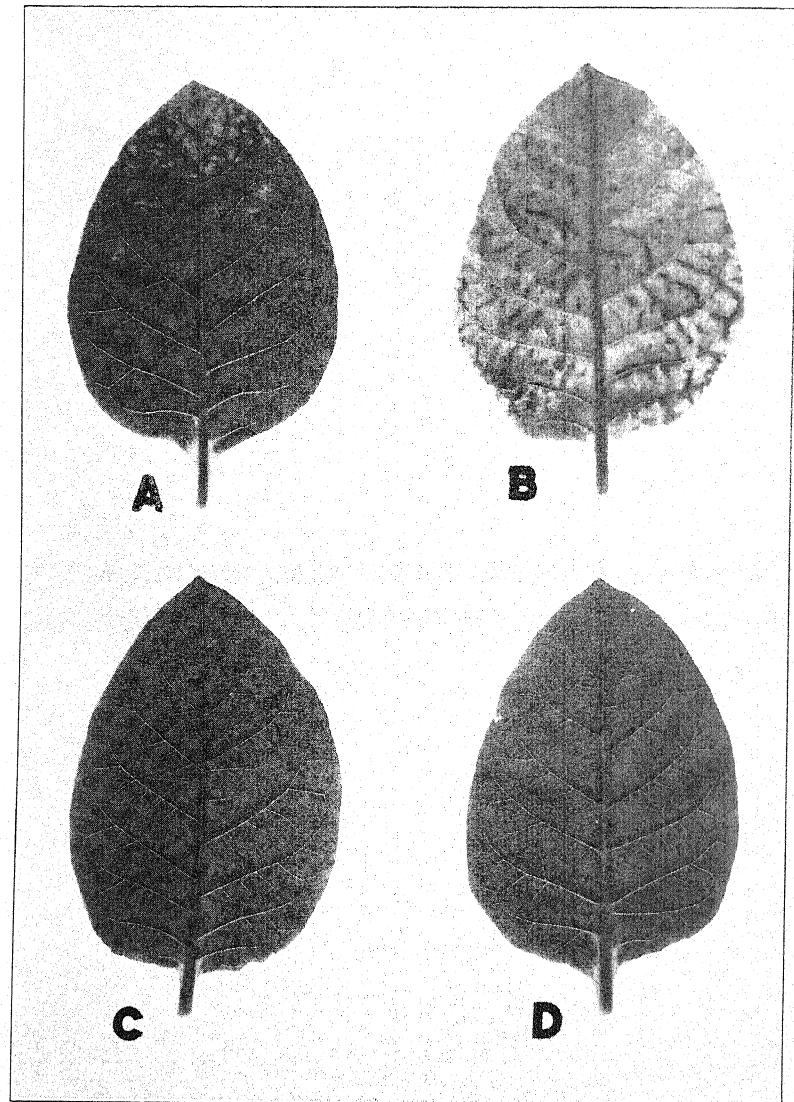

FIGURE 9. A, a typical Turkish tobacco leaf from a recovered ring-spot plant affected with tobacco mosaic; B, a similar leaf from a plant affected with tobacco mosaic only; C, a leaf from a recovered ring-spot plant; D, a leaf from a healthy control plant.

but became milder after ring-spot lesions were no longer produced on the tip leaves of these plants. The results of these experiments indicate that the presence of ring-spot virus interfered with the development of mosaic symptoms although ring-spot lesions could not be detected on new growth 14 days after inoculation with ring-spot virus.

DISCUSSION

A severe systemic disease is produced when certain species of plants are infected with ring-spot virus. This disease is characterized by the development of zonate necrotic spots on inoculated leaves as well as on leaves produced subsequent to inoculation. Leaves on which these spots occur are usually distorted and may also bear large irregular necrotic areas resulting from a complete breakdown of some of the venal and intervenal tissues. These irregular necrotic areas occur more frequently in some species than in others but zonate spots occur in all the species of *Nicotiana* which were tested by the writer.

Plants of susceptible species of *Nicotiana* invariably recover from the ring-spot disease under greenhouse conditions. Recovery from ring-spot occurs under different environmental conditions and is a normal behavior of many kinds of plants. It has been shown by inoculation tests that healthy-appearing leaves of recovered plants invariably contain ring-spot virus. In spite of the fact that considerable quantities of virus are present in recovered plants, they never develop the necrotic spots which are so characteristic of the disease. Furthermore, such symptoms are never produced on recovered plants by reinoculating them with virus shown to be virulent by inoculation of control plants. Each plant is susceptible to only one attack of the disease. It is therefore concluded that plants acquire an immunity to the ring-spot disease. It is also concluded that this immunity is lasting since it persists in plants grown through several generations by cuttings.

There is some evidence that the recovered plants grow more slowly than healthy plants but it has not been clearly demonstrated whether this difference in rate of growth is the result of the presence of virus or of the initial shock of the disease. Leaves of recovered plants appear to be slightly darker in color and of a somewhat more leathery texture than healthy controls. Such differences are insignificant when compared with the striking differences between recovered plants and plants suffering from an acute attack of the disease.

In some hosts, as *N. sylvestris*, recovery from ring-spot appears to be complete. That is, all the leaves produced by such plants after recovery are healthy in appearance and cannot be distinguished from healthy controls except by inoculation tests. Such plants appear to be completely immune to the ring-spot disease, even under the best growing conditions, except for

the fact that they harbor ring-spot virus. While it is possible that such plants may occasionally show disease symptoms under special environmental conditions, this has not been observed. Furthermore, the recovered plants of these species are immune to the disease under a wide range of conditions while healthy plants are susceptible under an equally wide range of conditions.

Plants of such varieties of *N. tabacum* as Burley and Turkish invariably recover from ring-spot but occasionally fail to completely recover. In such cases, the recovered plants show mild chlorotic or necrotic symptoms on the margins and tips of a few of their leaves. The necrotic areas may extend inward in an irregular fashion to the midrib. Such symptoms are not nearly so severe as and are easily distinguishable from those produced in the early stages of the disease. These plants are susceptible to one and only one acute attack of the disease.

From the results reported in this paper, it appears that the most significant way in which acquired immunity to ring-spot differs from recovery and acquired immunity in the animal kingdom is that ring-spot virus persists in the recovered plants. However, animals which recover from certain diseases are known to harbor the pathogens of these diseases. In fact, it is believed by some workers that acquired immunity to virus diseases is associated with the presence of virus in the immune animal. In only a few cases, though, has it been shown conclusively that virus persists in the animal after recovery. Cole and Kuttner (11) were able to obtain virus from the submaxillary glands of guinea pigs which were immune to the effects of this virus introduced elsewhere into their bodies. It was also shown by these workers that very young guinea pigs, which do not harbor the virus, are susceptible to infection and are often killed by intracerebral inoculations of this virus or will develop a local lesion if inoculated at any other site. Thus it appears that immunity to the disease is accompanied by the presence of virus in the immune animal. A similar case is the infectious anemia of horses. Kock (25) showed that a horse's blood was infectious as long as seven years after an attack of this disease. In this case the recovered horses are not completely immune, since they may suffer a relapse in some instances. Whether acquired immunity to ring-spot is of the same nature as acquired immunity in the animal kingdom is questionable but the writer believes that it is the nearest approach to this condition that has yet been observed in plants.

It has been shown conclusively that the primary factor involved in acquired immunity to ring-spot is an attack of the disease. This has been shown by the fact that plants which have recovered from ring-spot are immune to the disease, whereas, under the same conditions, healthy plants develop severe symptoms when infected with ring-spot virus. The question of why recovered plants of some varieties of *Nicotiana* occasionally develop

mild symptoms while recovered plants of other varieties do not show these symptoms under a wide range of conditions cannot be answered at this time. However, the tolerance of the host to virus after recovery does not appear to be correlated with the degree of tolerance shown by such plants previous to recovery. The onset of the disease is severe in *N. sylvestris*, as well as in Burley tobacco. Nevertheless, recovered plants of the former species are not distinguishable in appearance from healthy controls while in some instances recovered plants of the latter show distinguishable symptoms. Thus, it appears that the increase in defense mechanism in the host, resulting from an attack of the disease, is not necessarily dependent upon the defense mechanism existing in the host previous to infection.

Some workers (14, 18, 45) have referred to recovery in ring-spot as masking of the symptoms. It should be pointed out in this connection that masking has been used previously to describe plants in which a virus multiplies without producing marked symptoms of disease. Masked carriers are plants which carry a virus but show no marked symptoms under any conditions in which they have been grown. An example of this condition is furnished by the so-called healthy potato virus (24). This virus is present in many healthy-appearing potato plants but produces no marked symptoms in such plants. When transferred to healthy tobacco plants, however, it produces easily distinguishable symptoms of disease. Masking is also applied to the condition in which the disease symptoms disappear when plants are grown under special environmental conditions. This behavior has been demonstrated for tobacco mosaic (23) and for potato mosaic (16), among others. In such cases, however, masking is not permanent but lasts only as long as the plants are kept under these special conditions. Such plants again show characteristic symptoms when returned to the original environment. Recovery from ring-spot always follows a severe attack of the disease. Furthermore, the symptoms of ring-spot disappear under conditions which permit the development of severe symptoms in previously healthy plants. The recovered plants cannot be properly referred to the category of masked carriers.

It has been pointed out in another section of this paper that in no other disease has it been clearly demonstrated that recovered plants are immune to reinfection. It is probable that all the plants which recover from sugar cane mosaic are susceptible to reinfection (28) although their susceptibility has not been proved definitely. A similar situation apparently exists in the case of sugar cane plants which recover from the streak disease (39). It has been suggested but not conclusively demonstrated that plants which recover from sugar beet mosaic are virus carriers (35). Baur (1) reported that plants of *Abutilon striatum* infected with mosaic occasionally develop immune shoots and he also stated that he was unable to obtain virus from such shoots. While it is possible that abutilon plants may

acquire immunity to mosaic, Baur's evidence on this point is not conclusive.

Acquired immunity has been conclusively demonstrated for many diseases of animals. In this field, acquired immunity is closely associated with a normal recovery of the diseased animals. In plants, on the contrary, normal recovery occurs only rarely. Most of the diseases studied by plant pathologists either become chronic or result in death of the infected plants. In virus diseases of plants, recovery may occasionally occur but appears to be brought about by environmental conditions and is not recognized as a normal behavior of plants affected with these diseases. But ring-spot is a disease from which certain species of plants normally recover. Recovered plants or recovered animals need not necessarily be immune to the disease from which they have recovered. Nevertheless, it is only in the case of diseases from which individuals recover that one would expect to find acquired immunity in members of a susceptible race.

No evidence was obtained from grafting experiments conducted during the course of these studies that immunity was passively transferred to healthy plants. In fact, ring-spot is readily transmitted by intergrafting healthy and recovered plants under the conditions of the writer's experiments. If antibodies are present in recovered plants it might be expected that they would move to the growing tip and prevent the development of ring-spot symptoms on the young leaves. The results of grafting experiments, however, indicate that such is not the case. This evidence does not show that antibodies are not produced in recovered plants. It may be that such substances are confined to the cells in which they are produced and therefore have no effect on other cells of the host. Since the evidence indicates that antibodies do not move from one portion of the plant to another, it appears that the immunity acquired by ring-spot plants is a tissue immunity rather than a humoral one.

The question of whether ring-spot virus multiplies in recovered plants is an extremely interesting one. It may be that the virus does not multiply at all in recovered plants or multiplies less rapidly in the recovered plant than in the previously healthy plant. The evidence obtained by the writer from measurements of virus concentration in recovered plants and in plants during the onset of the disease are not conclusive. This evidence does suggest, however, that virus may be present in a lower concentration in recovered plants than in severely diseased plants. The fact that virus may be obtained in quantity from recovered plants grown through several generations by cuttings is not proof of the multiplication of virus in recovered plants. Such plants should be carried through many more vegetative generations in order to insure a high dilution of the virus which was produced during the onset of the disease. It is believed that a study of such phenomena as multiplication of virus may serve to throw some light on the

question of the mechanism by means of which plants recover from the ring-spot disease.

SUMMARY

1. The symptoms of ring-spot in Turkish tobacco vary with the environmental conditions. Inoculated plants kept in darkness develop water-soaked necrotic spots and are eventually killed by the disease. Inoculated plants grown under humid conditions develop spots consisting of many concentric rings; those under dry conditions develop spots consisting of few concentric rings. Plants grown in shade produce large numbers of spots consisting of fine necrotic rings. Ring-spot symptoms developed under all the environmental conditions which were tested.

2. Recovery from the ring-spot disease was observed in all the plants tested of four species of *Nicotiana* (*N. langsdorffi* Schrank., *N. sylvestris* Spegaz. & Comes, *N. quadrivalvis* Pursh., and *N. tabacum* L.) and of ten varieties of *N. tabacum*. This recovery was complete in some species and incomplete in others. Complete recovery consisted in the continued production of healthy-appearing leaves on plants which previously developed leaves with necrotic spots. Although most of the plants tested recovered completely from the disease, some plants of several varieties of *N. tabacum* showed incomplete recovery. Such plants occasionally produced leaves which were distinguished by slightly chlorotic or necrotic margins and inconspicuous, browned, distorted tips.

3. Juice from all the recovered plants tested was found to be highly infectious. The symptoms produced by inoculation of healthy tobacco plants with virus from recovered plants were identical with those produced by inoculation with virus from severely diseased plants. Thus, evidence was obtained that the virus of ring-spot was not attenuated in the recovered plants.

4. Symptoms were not produced on recovered plants by inoculating them with ring-spot virus although healthy plants grown under the same conditions developed severe symptoms when inoculated with the same virus samples. It is concluded that the four species of *Nicotiana* listed above acquired an immunity to ring-spot after infection with this disease.

5. In certain experiments, tobacco plants acquired immunity to ring-spot without showing symptoms of the disease. In these experiments, virus was inoculated into stem tissue of plants, the tip portions of which were kept defoliated until virus had reached the young tip bud.

6. It was shown that immunity to the ring-spot disease persisted in Turkish tobacco plants grown through three generations from cuttings but was not transmitted through seed to any of 825 seedlings tested.

7. No evidence was obtained from grafting experiments that acquired immunity in Turkish tobacco was accompanied by the production of antibodies.

8. The symptoms of tobacco mosaic in plants which recover from ring-spot are different from those in plants which have never had the ring-spot disease.

9. Certain species of plants, including beans (*Phaseolus vulgaris* L.) and cowpeas (*Vigna sinensis* Endl.), are killed by ring-spot.

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CELERY YELLOWS OF CALIFORNIA NOT IDENTICAL WITH THE ASTER YELLOWS OF NEW YORK

L. O. KUNKEL

INTRODUCTION

In a former paper the writer (6) reported experiments in which attempts were made to transmit aster yellows to celery (*Apium graveolens* L.) and zinnia (*Zinnia elegans* Jacq.). Since all trials were uniformly unsuccessful under conditions favorable for transmission to the China aster (*Callistephus chinensis* Nees) and other susceptible species such as lettuce (*Lactuca sativa* L.), strawflower (*Helichrysum arenarium* DC.), and buckwheat (*Fagopyrum esculentum* Moench) it was concluded that celery and zinnia are highly resistant or perhaps even immune to the aster yellows disease.

Severin (8) reported aster yellows as a serious disease of celery in California. He found it easy to transmit to celery, parsley, carrot, lettuce, zinnia, and aster plants by means of *Cicadula sexnotata* (Fall.),[1] the leafhopper which spreads aster yellows. As it was difficult to explain satisfactorily these contradictory results it was suggested that the yellows with which Severin worked in California might not be aster yellows.

During the summer of 1931 Severin kindly sent the writer yellowed specimens of aster, celery, and carrot. The plants were received in fairly good condition and remained alive long enough to be used in transmission tests. It thus became possible to compare the yellows from California with the aster yellows of New York. The object of this paper is to report the results of the comparison.

TRANSMISSION EXPERIMENTS

No difficulty was experienced in transmitting yellows from the diseased aster, celery, and carrot plants obtained from California to healthy aster plants by means of *Cicadula sexnotata*. The disease thus produced showed symptoms typical of aster yellows and could not be distinguished from the yellows disease prevalent on asters in New York. The first symptoms appeared in from 12 to 18 days after exposure to virus-transmitting leafhoppers. The incubation period of the California disease in the aster plant is approximately the same in length as was found for the New York disease under similar conditions.

No zinnia plants and only a few celery plants were available for experi-

[1] On the basis of a comparison of American specimens of *Cicadula sexnotata* with specimens from Russia identified as *C. sexnotata*, Dorst (2) has referred the American specimens to the species *C. divisa* Uhler. *Cicadula divisa* Uhler is considered by some entomologists to be distinct from the American species which has been known as *C. sexnotata*. In view of this uncertainty, the writer has retained the name *C. sexnotata* for the vector of aster yellows.

mental purposes at the time the California yellows was received. In order to test the possibility of transmitting this disease to celery under New York conditions, colonies of 100 virus-free nymphs of our *Cicadula sexnotata* were placed on a healthy aster plant and on each of three aster plants having the California disease. After being confined on the plants for seven days the four insect colonies were placed on four different healthy young celery plants of the variety Silver Self Blanching for two weeks. The insect colonies were then removed and destroyed. The three celery plants exposed to colonies which had fed on yellowed aster plants began to show the

FIGURE 1. Two celery plants of the variety Silver Self Blanching. The plant on the right was exposed to a large virus-free colony of *Cicadula sexnotata* and is healthy. The plant on the left was exposed to an equally large colony of the same leafhopper carrying the virus of the California yellows disease. This plant shows typical symptoms of the disease

symptoms of celery yellows as described by Severin (8) after a period of about four weeks from the time when they were first exposed. The celery plant exposed to the colony which had fed on a healthy aster plant during the time the other three colonies were on yellowed plants remained healthy. Figure 1 shows this plant and one of the celery plants which took the yellows disease. The experiment proved that the yellows from California

can be transmitted to celery plants of the variety Silver Self Blanching by insect colonies similar to those that were unable to transmit the aster yellows of New York to plants of the same variety in experiments previously reported (6).

Yellows was transferred from one of these experimentally-infected celery plants back to aster plants.

When it became evident that the California yellows differs from the aster yellows of New York with respect to transmission to celery, an attempt was made to determine whether it might not also differ with respect to its incubation period in the insect vector. One hundred virus-free leafhoppers were confined on an aster plant having the California yellows for a period of five days. Twenty healthy young aster plants of the variety Semple's Late Flowering were then exposed on successive days to this colony of insects for a period of one day each. After exposure the plants were placed in a greenhouse kept free from insects by fumigation. The first 13 plants exposed to this colony of insects remained healthy during a period of two months that they were kept under observation. All of the seven plants exposed after the thirteenth day following removal of the insect colony from the yellowed aster plant took the yellows disease. A period of 18 days elapsed between the beginning of the five-day period of feeding on the yellowed plant and the time when the colony was first able to transmit the virus. If it be assumed that the insect picked up the virus during the first day of feeding on the yellowed plant, then an incubation period of 18 days in the insect is shown by the test.

In a similar experiment 125 virus-free leafhoppers were confined for five days on a carrot plant having the California yellows. They were then allowed to feed on successive days for a period of one day on each of 20 healthy young aster plants of the variety Heart of France. The plants on which the leafhoppers fed during the first 12 days following their removal from the carrot plant remained healthy. All of the eight plants on which they fed after the twelfth day following their removal from the carrot plant took the yellows disease. A period of 17 days elapsed between the time when the insects first fed on the yellowed carrot plant and the time when they were first able to transmit the disease. An incubation period of 17 days in the insect is shown by the test. These experiments demonstrate that the incubation period of the California yellows virus in the insect vector, *Cicadula sexnotata*, is similar in length to that shown by the virus of the aster yellows of New York in the same insect. The greenhouse in which these tests were made was held at a minimum temperature of 70° F. At higher temperatures the incubation period might have been much shorter.

As soon as it was possible to obtain a sufficient number of healthy young celery plants a second transmission experiment was undertaken. A few zinnia plants were included in the test. The plan of the experiment was

TABLE I

HOST RANGES OF THE ASTER AND CELERY YELLOWS DISEASES

Days on which plants were exposed	14th	15th & 16th	17th & 18th	19th	20th	21st	22nd	23rd & 24th	25th	26th
Kind of plants exposed	Aster	Aster	Aster	Aster	Aster	Celery	Aster	Celery	Aster	Celery
No. 1 Insect culture carrying yellows from California	+	+	+	‡	‡	+	‡	‡	‡	‡
No. 2 Insect culture carrying yellows from California	+	+	+	+	+	+	‡	‡	‡	‡
No. 3 Insect culture carrying yellows from California	+	+	+	+	+	+	+	+	+	‡
No. 4 Insect culture carrying yellows from California	+	+	+	+	+	+	+	+	+	‡
No. 5 Insect culture carrying yellows from California	+	+	+	+	+	+	+	+	‡	‡
No. 6 Insect culture carrying yellows from California	+	+	+	+	+	+	+	‡	‡	+
No. 7 Insect culture carrying aster yellows of New York	+	+	+	‡	‡	+	‡	+	‡	+
Check culture	+	+	+	+	+	+	+	+	+	+

TABLE I (*Continued*)

HOST RANGES OF THE ASTER AND CELERY YELLOWS DISEASES

Days on which plants were exposed	27th	28th	29th	30th	31st	32nd & 33rd	34th	35th	36th & 37th	38th & 39th	40th
Kind of plants exposed	Aster	Zinnia	Aster	Zinnia	Aster	Celery	Aster	Zinnia	Celery	Aster	Celery
No. 1 Insect culture carrying yellows from California	+	+	‡	+	‡	‡	‡	+	+	+	+
No. 2 Insect culture carrying yellows from California	‡	+	‡	+	‡	‡	‡	+	‡	‡	+
No. 3 Insect culture carrying yellows from California	+	+	‡	+	‡	‡	‡	+	+	‡	+
No. 4 Insect culture carrying yellows from California	‡	+	‡	+	‡	+	‡	+	‡	–	–
No. 5 Insect culture carrying yellows from California	‡	+	‡	+	‡	‡	‡	+	‡	‡	+
No. 6 Insect culture carrying yellows from California	‡	+	‡	+	‡	‡	‡	+	+	–	–
No. 7 Insect culture carrying aster yellows of New York	‡	+	‡	+	‡	+	‡	+	+	‡	+
Check culture	+	+	+	+	+	+	+	+	+	+	+

The plus sign indicates that plants remained healthy, the double plus sign that they became diseased. The minus sign indicates that no plants were exposed on that day. The table shows that celery yellows was readily transmitted to celery and aster but not to zinnia plants. Aster yellows was readily transmitted to aster but not to celery or zinnia plants.

to expose simultaneously celery, zinnia, or aster plants of the same ages and varieties held under the same conditions of light, temperature, and humidity to colonies of leafhoppers carrying the California yellows, to a colony of leafhoppers carrying the aster yellows of New York, and to a check colony of virus-free leafhoppers.

Six colonies consisting of 30 virus-free nymphs each and designated by the numbers 1 to 6 were placed on each of 6 aster plants having the California yellows disease. A similar colony designated as number 7 was placed on an aster plant having the aster yellows of New York, and another similar colony, to be used as a check, was placed on a healthy aster plant. The colonies remained on the plants for one week. They were then confined on healthy young aster plants for six days. After the thirteenth day following that on which the experiment was started all colonies were transferred daily or at intervals of two days to healthy young aster, zinnia, or celery plants. The aster plants used were of the variety Semple's Late Flowering, the zinnia plants of the variety Giant Double Dahlia Flowering, and the celery plants were of the variety White Plume. The experiment was continued over a period of 40 days. The colonies were confined on celery plants during the twenty-first, twenty-fourth, twenty-sixth, thirty-second, thirty-third, thirty-sixth, thirty-seventh, and fortieth days of the experiment. They were on zinnia plant during the twenty-eighth, thirtieth, and thirty-fifth days. On all other days they were confined on aster plants. After being exposed, all plants were placed in a greenhouse which was fumigated at frequent intervals to kill nymphs that hatched from eggs deposited in them. All plants were held under observation for two months after the experiment was ended. The results obtained are shown in Table I.

The experiment shows that the aster yellows of New York is not transmitted to the celery variety White Plume by *Cicadula sexnotata*. This result confirms conclusions reached from previous work (6). The yellows from California is readily transmitted to celery of the variety White Plume.

The table shows that the incubation period of the virus was not completed in any of the colonies carrying the California disease before the nineteenth day after the experiment was begun. It was apparently not completed in colony 2 until the twenty-second day, in colonies 3 and 4 until the twenty-sixth day, in colony 5 until the twenty-fifth day, and in colony 6 until the twenty-third or twenty-fourth day. This would account for failure of transmission to 34 of the aster plants exposed from the fourteenth to the twenty-fifth day of the experiment. Colonies 1 and 2 were old and weak by the thirty-eighth day. This would account for their failure to transmit yellows to the two plants on which they fed during the thirty-eighth and thirty-ninth days. The California yellows was transmitted to 32 of the 34 aster plants exposed after the completion of the incubation

period and before the colonies became old and weak. Aster yellows was transmitted to all of the 9 aster plants exposed to colony 7 after the completion of the incubation period in this colony on the nineteenth day of the experiment.

Yellows was transmitted to 16 of the 34 celery plants exposed to leafhopper colonies carrying the California disease. Colony 1 transmitted it to three of the six celery plants exposed, colony 2 to four, colonies 3, 4, and 6 to two plants each, and colony 5 to three plants. The virus had apparently not completed its incubation period in colonies 2, 3, 4, 5, and 6 when the first set of celery plants was exposed. It had not completed its incubation period in colonies 3, 4, and 5 when the second set of celery plants was exposed.

FIGURE 2. Three celery plants of the variety White Plume. They were exposed for one day to three similar colonies of *Cicadula sexnotata*. The plant on the left was exposed to a virus-free colony, that in the middle to a colony carrying the aster yellows of New York and infective to asters. Both plants are healthy. The plant on the right was exposed to a colony carrying the yellows from California and is diseased.

posed. This would account for failure of transmission to eight of the celery plants exposed on the twenty-first, twenty-third, and twenty-fourth days of the experiment. Three of the colonies failed to transmit yellows to the aster plants on which they fed during the thirty-eighth and thirty-ninth days of the experiment. It is not surprising that they failed to transmit it to celery on the following day. Colonies 4 and 6 were dead and all other colonies had dwindled in numbers by the fortieth day. This probably accounts for their failure to transmit yellows to the celery plants exposed during the last day of the experiment. The colonies carrying the California disease transmitted yellows to 16 of the 21 celery plants on which they fed after the completion of the incubation period of the virus and before the colonies

became old and weak. The disease was transmitted to about three-fourths of the celery plants exposed during the time the colonies were transmitting freely to aster plants. Leafhopper colonies that were able to transmit the disease to aster and celery failed to transmit it to zinnia.

The aster yellows of New York was not transmitted to any of the six celery plants exposed to colony 7 although this colony transmitted it to all of the nine aster plants on which it fed immediately before and during alternate days of the period over which the celery plants were exposed. Figure 2 shows three of the celery plants exposed on the twenty-sixth day of the experiment and illustrates the difference in the behavior of the two diseases. The photograph was taken 43 days after the plants were exposed to leafhopper colonies.

In a further test the yellows from California was transmitted to three celery plants of the variety Golden Self Blanching. Attempts to transmit it to three healthy young zinnia plants by the same insects used to transfer it to these celery plants were unsuccessful.

Neither aster yellows nor the yellows from California was transmitted to zinnia. This is rather surprising since Severin found zinnia to be a common host of yellows in California.

DISCUSSION

The experiments here reported suggest an explanation of why celery fields in New York and other eastern states are free of yellows of the virus type whereas the fields of California are seriously injured by the yellows disease prevalent in that state. The aster yellows of New York has not been transmitted to celery under conditions that are favorable both for its transmission to the aster and other susceptible plants and for the transmission of the yellows from California to celery. It is concluded that celery is highly resistant or perhaps even immune to the aster yellows of New York. Whether the yellows from California is a strain of aster yellows or is a different disease is a question that can not be answered at this time. It is not uncommon for two different virus diseases to have overlapping host ranges or to produce similar symptoms. On the other hand diseases which appear to be strains of certain other virus diseases have not been shown to have different host ranges. Yellow mosaic of tobacco described by Johnson (5) and aucuba mosaic of tomato studied by Smith (9) are so similar as regards the properties of their causative agents as to suggest that they may be strains of the tobacco mosaic disease. Johnson (4) obtained attenuated strains of tobacco mosaic by exposing inoculated tobacco plants to a constant temperature of 35° C. to 37° C. for 10 or more days. The attenuated strains as well as the strains occurring naturally are distinguishable by differences in symptoms. They are not known to show host range differences.

Carsner (1) has reported the natural occurrence and experimental production of an attenuated strain of the curly top of sugar beets. He obtained it by passage of the virulent form of curly top through *Chenopodium murale* and certain other weeds. It causes a mild form of the curly top disease in beets. Its host range apparently coincides with that of the virulent form. If the yellows from California is a strain of the aster yellows disease it is obviously a more distinct variant than the known strains of tobacco mosaic or the attenuated strain of the curly top disease. The readiness with which it goes to zinnia in California brings additional evidence in favor of this view. Further studies on the California disease, especially studies on its host range, may prove that it differs in other ways from aster yellows. The fact that both are transmitted by the same insect vector, have long incubation periods, and produce similar symptoms in asters and some other plants, supports the view that they may be related.

In 1927 Linford (7) reported a yellows on celery in Utah. The symptoms observed indicated that it might be a virus disease but Linford states that this is not supported by the field occurrence of the disease. Folsom (3) found a yellows on celery in southwestern Maine which he says is apparently the same disease as that described by Severin. There is, however, no convincing evidence that either this or the disease reported by Linford is the California yellows. In so far as the writer is aware no disease resembling that described by Severin has been observed in other celery-growing regions of the United States. Careful observation of several celery plantings in the vicinity of New York City during the past three seasons has failed to reveal its presence. Since the California yellows is a serious disease of celery and causes symptoms that are not easily overlooked, it should have been observed if it were of general occurrence outside of California. Whether a disease identical with the aster yellows of New York is prevalent in California is not known. Aster plants in California are apparently less seriously injured by yellows than are those in the eastern states. This suggests that California may be free of the aster yellows disease common throughout the east. Severin (8) states that yellowed asters were first observed in California in 1925. This indicates that the disease now prevalent in that state is of recent introduction.

SUMMARY

1. A yellows disease obtained from California was transmitted from aster, celery, and carrot plants to aster plants by the leafhopper *Cicadula sexnotata*.

2. It was readily transmitted from aster to celery plants of the varieties Silver Self Blanching, White Plume, and Golden Self Blanching by colonies of *Cicadula sexnotata*.

3. Similar colonies were unable to transmit the aster yellows of New York to celery.

4. The disease from California was transferred from an experimentally-infected celery plant to aster plants. It could not be distinguished through symptoms produced in asters from the aster yellows of New York.

5. In two tests of the length of time required for the incubation of the virus of the disease in the vector, periods of 17 and 18 days respectively were shown. In six other tests incubation periods of from 19 to 26 days were shown. These periods do not differ much in length from those found for the aster yellows of New York.

7. The yellows from California differs from the aster yellows of New York in respect to transmission to celery. Other differences have not yet been demonstrated.[2]

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[2] The manuscript of a paper comparing the aster yellows of New York with the yellows prevalent on celery in California was received from Dr. H. H. P. Severin after this paper was submitted for publication.

THE FUNGICIDAL ACTION OF SULPHUR. IV. COMPARATIVE TOXICITY OF SULPHUR, SELENIUM, AND TELLURIUM[1]

Frank Wilcoxon and S. E. A. McCallan

In the second paper of this series (6), evidence was presented that the toxicity of sulphur toward certain pathogenic fungi is due to an interaction of the sulphur vapor with the fungous spores resulting in the reduction of sulphur to toxic hydrogen sulphide. In the hope of throwing further light on this subject it was decided to investigate the comparative toxicity of sulphur and the analagous elements, selenium and tellurium, of group VI of the Periodic System.

Wöber (13) has discussed the toxicity toward *Plasmopara viticola* Berl. & de Toni of the elements in relation to their position in the periodic table. He, however, dealt only with the metals and did not discuss sulphur, selenium, and tellurium. Recently Lougee and Hopkins (4) and Stover and Hopkins (9) made a study of selenium and tellurium compounds as possible fungicides to control certain fruit and tree diseases. Their results were not encouraging from a practical standpoint because of injury to the foliage.

If the action of elementary selenium and tellurium on fungous spores is similar to that of sulphur then the factors which would be important in determining its toxicity are the vapor pressure, the ease of reduction to the hydrides, and the toxicity of these hydrides to fungous spores. While precise values for the vapor pressures of solid sulphur, selenium, and tellurium are not available, nevertheless from the vapor pressures of the liquids at higher temperatures and from the melting points, it is apparent that the vapor pressure of the elements in the solid state must lie in the following descending order: sulphur, selenium, and tellurium.

From calculations of the free energy of formation of the hydrides it may be predicted that sulphur would be reduced to hydrogen sulphide with relative ease, selenium to hydrogen selenide less easily, while the reduction of tellurium to hydrogen telluride would be the most difficult of all. In agreement with this order it has been shown by Bergstrom (1) that sulphur will liberate selenium from selenides in liquid ammonia solution, and that selenium will displace tellurium from tellurides. The stability of the hydrides falls in the reverse order, hydrogen sulphide being the most stable of the three and hydrogen telluride the least stable (8, p. 38). From these considerations, elementary selenium and tellurium would be expected to be less toxic than sulphur when compared under similar conditions even though the toxicity of the hydrides were the same.

[1] Herman Frasch Foundation for Research in Agricultural Chemistry, Paper No. 49.

METHODS

The toxicity has been determined by the moist chamber method of spore germination used and described in previous studies (5, 6, 11, 12). The spores of three species of fungi have been employed. Two of these species, *Sclerotinia americana* (Worm.) Nort. & Ezek. and *Uromyces caryophyllinus* (Schr.) Wint., are sulphur-sensitive, while the third, *Pestalotia stellata* B. & C., is relatively sulphur-resistant (6). The two latter species were from the same source as those described in the second paper of this series (6), while the *Sclerotinia* was from two sources. The first was a reisolation from the same cherry tree (*Prunus avium* L.) that furnished the strain used previously (6, 11, 12), while the second, an isolation from plum (*Prunus* sp.) at Ithaca, New York, was obtained through the courtesy of Prof. H. H. Whetzel. Unless otherwise stated in the following pages the former was used.

The germination factors, as regards age of spores, germination medium, temperature and time were similar to those formerly reported (12). A factor often given too little attention is that of the density of the spore suspension. In an experiment performed to determine the effect of density of spore suspension on the toxicity of sulphur it was found that percentage germination was positively correlated with spore density. This experiment comprised 200 observations totalling 7570 spores with varying amounts of sulphur dust. The value of the coefficient of partial correlation obtained was 0.239, the probability of significance being greater than 100 to 1. Accordingly, an effort has been made to have the spore density as constant for a single experiment as is possible. A density allowing an approximate distribution of 10, 15, or 25 spores per square millimeter has been used in the various experiments.

In the majority of experiments reported in this paper, comparisons of sulphur, selenium, and tellurium have been made by means of toxicity curves. The concentration of toxic agent has been plotted as numbers of particles per square millimeter since, as the authors have shown (12), the toxicity of sulphur is dependent on the number of particles. To obtain these concentrations, slides were dusted with varying quantities of dust and at the time the germination counts were made, the number of particles in each field was recorded. The results were then arranged in order according to particle numbers in groups of five or eight determinations. Thus each point on a toxicity curve is the average of five or eight observations.

From 200 to 360 spores have been counted for each point on the toxicity curves. The authors have recently shown (7) that in experiments of this type the distribution of germinated and ungerminated spores is that to be expected from the variations of random sampling as shown by the χ^2 test. Hence from Table III of the above paper (7) it is seen that, where 300

spores are counted, the difference in per cent germination required for significance with odds of 50 to 1 is 9.5 at 50 per cent germination, and 6.8 at 85 per cent germination. For an entire curve, where a series of points is available for comparison, even less difference would be significant.

TESTS OF COMPARATIVE TOXICITY

SULPHUR, SELENIUM, AND TELLURIUM DUSTS

In these experiments two straight commercial sulphur dusts were used which did not contain any appreciable amount of impurities or added substances.

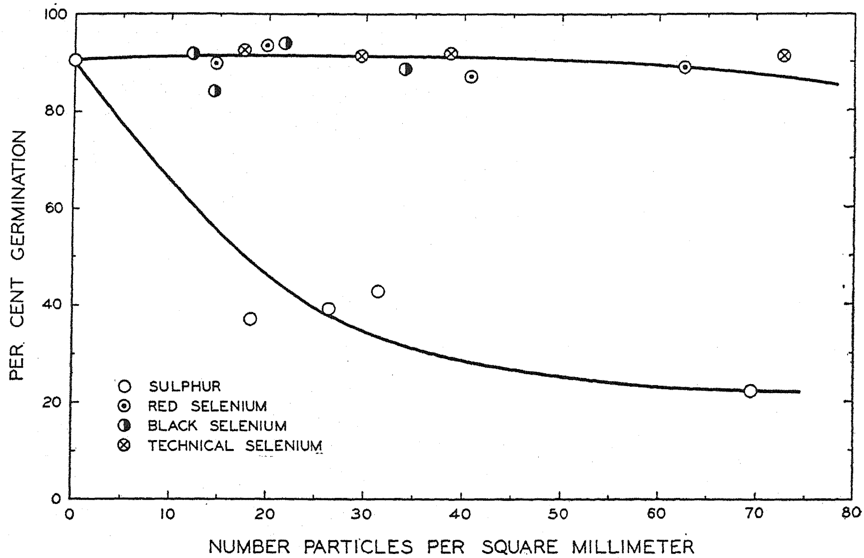

FIGURE 1. The comparative toxicity of sulphur dust and red, black, and technical selenium dusts to the conidia of *Sclerotinia americana* (Plum isolation).

The powdered selenium was obtained from a chemical supply house. A portion of this was purified by oxidation to the dioxide; the dioxide was sublimed, the sublimate was dissolved in water containing hydrochloric acid and the elementary selenium precipitated by passing a current of sulphur dioxide through the solution (3, p. 7). When precipitated cold the red amorphous selenium is obtained, which will be referred to below as red selenium. From a hot solution a darker product is obtained, referred to as black selenium. The unpurified product will be designated as technical selenium. There was no appreciable difference in toxicity between these three types of selenium as will be seen in Figure 1.

The tellurium was obtained in the form of cast sticks which were ground in a mortar, sieved and used without further purification.

The average particle diameter of the dusts varied from 6 to 15μ in the different experiments. However, it made no difference whether the particle sizes of the dusts undergoing comparison were the same or not, provided the number of particles per unit area was the same. This is in accord with work previously reported for sulphur (12).

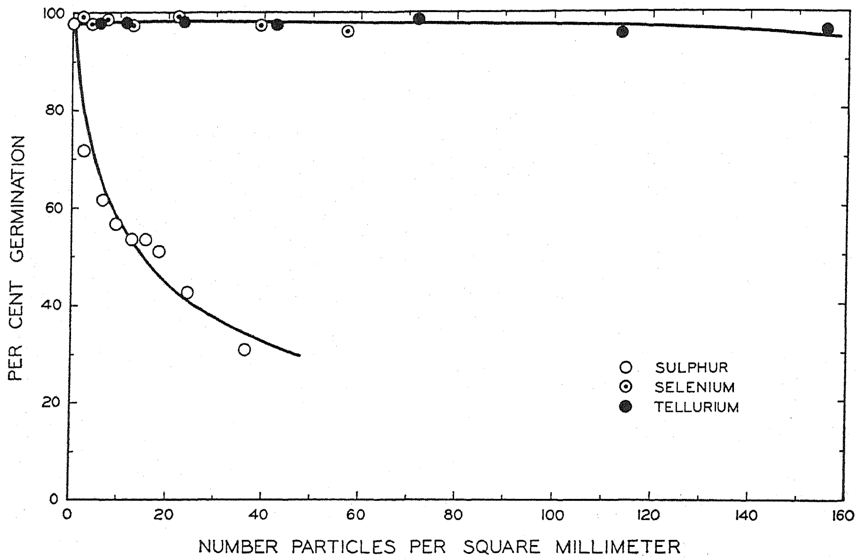

FIGURE 2. The comparative toxicity of sulphur, selenium, and tellurium dusts to the conidia of *Sclerotinia americana*.

The results of the comparative toxicity tests of the three elements sulphur, selenium, and tellurium on *Sclerotinia americana*, *Uromyces caryophyllinus*, and *Pestalotia stellata* are illustrated in Figures 2, 3, and 4.

The most striking fact that appears from inspection of these curves is the low toxicity of selenium and tellurium as compared to sulphur. In fact, as far as elementary selenium and tellurium are concerned they seem to offer little possibility for practical use as fungicides. Within the range of concentration studied there is no significant difference between the toxicity of selenium and tellurium. It will be noted that in the case of the relatively sulphur-resistant fungus, *Pestalotia stellata*, there is little difference between the toxicity of sulphur on the one hand and that of selenium and tellurium on the other. There was apparently slight stimulation by selenium and tellurium in the case of *Pestalotia stellata* and *Uromyces caryophyllinus*.

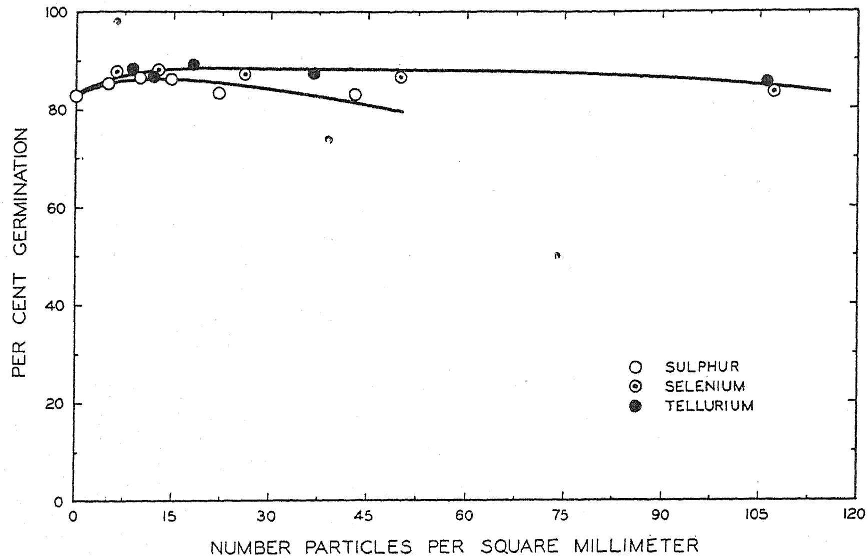

FIGURE 3. The comparative toxicity of sulphur, selenium, and tellurium dusts to the conidia of *Pestalotia stellata.*

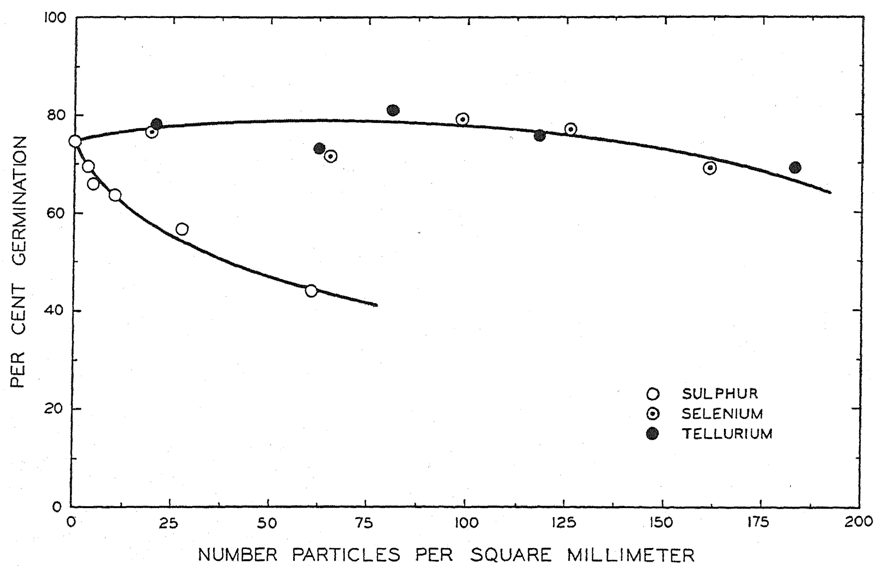

FIGURE 4. The comparative toxicity of sulphur, selenium, and tellurium dusts to the uredospores of *Uromyces caryophyllinus.*

COLLOIDAL SULPHUR AND COLLOIDAL SELENIUM

Since colloidal sulphur has been shown to be more toxic than ordinary ground sulphur (11, 12) it was considered possible that colloidal selenium might be much more toxic than the powdered element. Accordingly a comparison was made between colloidal sulphur and colloidal selenium solutions on a weight basis.

The colloidal sulphur was prepared by the addition of a small amount of an alcoholic solution of sulphur to a large volume of water (10). Colloidal selenium was prepared by reduction of a solution of selenious acid with dilute hydrazine hydrate (2). The selenium sol was dialyzed in collodion bags to remove soluble impurities.

TABLE I

COMPARATIVE TOXICITY OF COLLOIDAL SULPHUR AND COLLOIDAL SELENIUM TO THE CONIDIA OF SCLEROTINIA AMERICANA

Concentration, per cent by weight	Colloidal sulphur		Colloidal selenium	
	Per cent germination	Germ tube length μ	Per cent germination	Germ tube length μ
Control	96.5	350	96.5	350
0.0004	67.0	160	—	—
0.001	17.3	90	—	—
0.002	4.1	50	—	—
0.004	0	0	96.4	90
0.010	—	—	96.5	90
0.016	—	—	93.2	50
0.020	—	—	87.9	30

The average results of several experiments, using conidia of *Sclerotinia americana*, are given in Table I. It will be seen that the colloidal sulphur is much more toxic than the colloidal selenium. However, in the case of the colloidal selenium solutions at concentrations which produce no reduction in percentage germination there is a marked reduction in germ tube length.

Similar experiments were performed with conidia of *Pestalotia stellata* and in this case also the colloidal sulphur was more toxic than the colloidal selenium, although the difference was less marked since this is a relatively sulphur-resistant species.

HYDROGEN SULPHIDE AND HYDROGEN SELENIDE

Since elementary selenium, even in colloidal form, showed very little toxicity, it appeared to be of interest to determine the toxicity of its reduction product, hydrogen selenide, and to compare the latter with hydrogen sulphide. It is difficult, however, to work with hydrogen selenide in a quantitative manner, owing to its marked instability and tendency to undergo oxidation rapidly. Whereas, a moist chamber to which hydrogen

sulphide has been added will still contain appreciable quantities of the gas after a number of hours; in the case of hydrogen selenide the oxidation of the gas takes place in a few minutes. It is therefore necessary to provide for the continuous evolution of fresh quantities of the gas, in order to maintain a concentration sufficient to exert a toxic effect on the spores during their exposure.

In the following experiment small crucibles containing weighed amounts, respectively, of aluminum sulphide and aluminum selenide, were introduced into the usual moist chambers[8] containing spores of *Sclerotinia americana* in drops of water on slides. The atmospheric moisture in the chambers was sufficient to cause a slow hydrolysis of the aluminum compounds resulting in a slow liberation of hydrogen sulphide and hydrogen selenide respectively. Within a few minutes red colloidal selenium which was derived from the hydrogen selenide began to appear on the walls of the chambers and on the slides. In spite of the rapid decomposition of the gas, the concentrations within the chambers were sufficient to produce a markedly toxic effect, as is shown in Table II.

TABLE II
COMPARATIVE TOXICITY OF HYDROGEN SULPHIDE AND HYDROGEN SELENIDE, FORMED FROM THEIR RESPECTIVE ALUMINUM SALTS, TO THE CONIDIA OF SCLEROTINIA AMERICANA

Milligrams of aluminum salt	Per cent germination	
	Hydrogen sulphide	Hydrogen selenide
Control	96.8	96.8
0.75	0.1	46.3
5	0	39.3
25	0	0

Due to the fact that the rate of decomposition of hydrogen selenide is greater than that of hydrogen sulphide, it is probable that the concentration of selenide was less than that of sulphide during the period of germination.

It was thought that the apparent toxicity of hydrogen selenide might be due to the rather large amounts of colloidal selenium formed by its decomposition. In order to test this point a direct comparison was made between colloidal selenium prepared as previously described and the mixture of hydrogen selenide and colloidal selenium as obtained in these last experiments. The results of this comparison are illustrated in Figure 5, in which percentage germination is plotted against the density of colloidal selenium particles in each case. It will be observed that the colloidal selenium alone is much less toxic than the colloidal selenium where hydrogen selenide is also present. There seems no doubt, therefore, that hydrogen selenide like

hydrogen sulphide is very toxic to the spores of the fungi used, while elementary selenium is far less toxic than elementary sulphur.

Reduction of sulphur, selenium, and tellurium to form hydrides. The comparative extent to which sulphur, selenium, and tellurium undergo reduction in the presence of glutathione and of spores was tested in the following manner. Three small glass vials containing a water paste of powdered sulphur, selenium, and tellurium, respectively, were closed with stoppers bearing strips of lead acetate paper. To each of these vials was added, in one experiment, equal amounts of reduced glutathione, and in a second experiment, equal amounts of a suspension of yeast cells. After an hour the

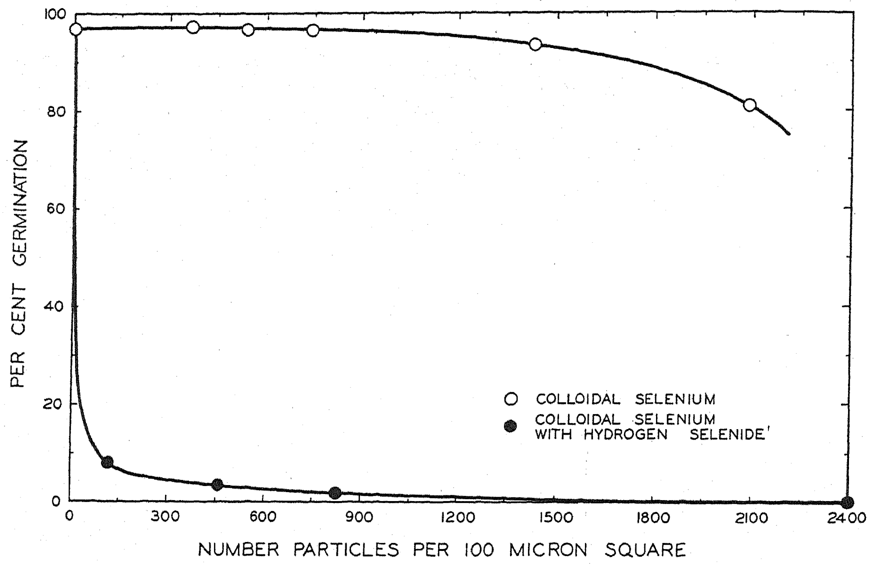

FIGURE 5. The comparative toxicity of colloidal selenium and a mixture of colloidal selenium and hydrogen selenide to the conidia of *Sclerotinia americana*.

difference in coloration of the paper in the three cases was very apparent. The lead paper in the vial containing sulphur started to blacken within a few minutes, while the vial containing selenium required several hours for an appreciable blackening to take place, and in the case of tellurium only the faintest darkening was visible after 24 hours. The result with the yeast cells is illustrated in Figure 6. This behavior offers a possible explanation for the low toxicity of elementary selenium and tellurium as compared to sulphur, and indirectly supports the explanation previously presented by the authors (6) for the mechanism by which sulphur exerts its toxicity. If the vapor pressure of selenium and tellurium is insufficient to permit appreciable quantities of these elements to reach the fungous spores, and

furthermore if the tendency towards reduction of these elements is less than in the case of sulphur, then they could scarcely be expected to be as toxic as sulphur.

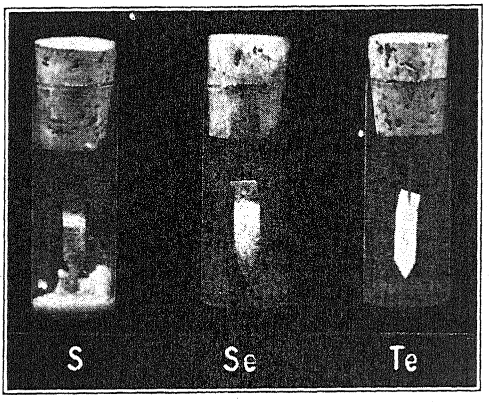

FIGURE 6. The comparative reduction of sulphur, selenium, and tellurium to the respective hydrides, by yeast spores, as indicated by the blackening of lead acetate paper.

SUMMARY

1. In order to throw further light on the mode of action of sulphur as a fungicide, which is believed to be due to the reduction of sulphur vapor to toxic hydrogen sulphide by the fungous spores, a study was made of the comparative toxicity of sulphur and its analogous elements, selenium and tellurium, of group VI of the Periodic System.

2. The comparative toxicity of sulphur, selenium, and tellurium dusts was measured using spores of *Sclerotinia americana*, *Pestalotia stellata*, and *Uromyces caryophyllinus*. It was found that selenium, both purified and technical, and tellurium were much less toxic than sulphur. Selenium even in colloidal form showed no appreciable toxicity.

3. The toxicity of hydrogen selenide, however, while difficult to measure accurately because of its instability, appeared to be comparable to that of hydrogen sulphide.

4. The formation of hydrogen selenide and of hydrogen telluride by yeast cells or by glutathione took place less readily than the reduction of sulphur to hydrogen sulphide under similar conditions.

5. These experiments indicate that elementary selenium and tellurium do not offer much promise as fungicides.

6. The results are in accord with the theory previously presented regarding the fungicidal action of sulphur.

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ECOLOGICAL AND PHYSIOLOGICAL STUDIES ON CERTAIN AQUATIC ANGIOSPERMS

W. S. Bourn[1]

INTRODUCTION

The inland waters of North Bay and Back Bay in Virginia and Currituck Sound in North Carolina (Fig. 1) have long been known to be one of the most important winter feeding grounds for migratory wild fowl in the United States. In these waters there formerly thrived in great abundance such submerged angiosperms as *Potamogeton pectinatus* L., *Potamogeton perfoliatus* L., *Potamogaton foliosus* Raf., *Naias flexilis* (Willd.) Rostk. & Schmidt, *Vallisneria spiralis* L., *Ruppia maritima* L., and *Ceratophyllum demersum* L., all being valuable food plants, according to McAtee (46, 47, 48, 50), for wild ducks, geese, and swan. In 1918, almost simultaneously with the opening and enlargement of the Albemarle and Chesapeake Canal, however, these plants began to die out, and by the end of 1926 vast areas were practically denuded of their aquatic seed plants. This destruction of the plant life produced an enormous economic loss affecting thousands of the residents, who derived their living from gunning and fishing. Shooting clubs and sportsmen practically abandoned their large investments in the region because wild ducks and geese in any appreciable numbers were no longer attracted there. At the request of Mr. William E. Corey, a prominent sportsman of New York, who had been interested in the region for many years, the Boyce Thompson Institute for Plant Research in 1925 undertook a study of the causes for the disappearance of the duck-food plants, and of methods of re-seeding depleted areas. Early the following year the writer was assigned to the investigation.

Studies were made continuously from 1926 to 1930, inclusive, of the physical, chemical, and biological conditions of the water. During this time ecological experiments were carried on for the purpose of determining varieties of plants resistant to existing conditions and to determine the best methods of re-seeding the barren areas.

The ecological investigations led naturally to physiological experiments in the laboratory to determine the factors limiting the growth of submerged angiosperms. These experiments have been continued to the present time.

REVIEW OF LITERATURE

A search through the literature has revealed no experimental work on submerged angiosperms in relation to their growth in brackish water. McAtee (46, 47, 48), writing on the importance of most of the plants men-

[1] The writer is indebted to Professors S. F. Trelease and R. A. Harper of Columbia

tioned as duck-food, reports the ability of a few to grow in brackish water, but he does not record definite limits of salt which the plants can endure. More definitely, Metcalf (53) concludes, from a survey of the duck-food plants growing in the alkaline lakes of North Dakota, that *Potamogeton perfoliatus* and *Ceratophyllum demersum* cannot withstand a salt content of the water greater than 0.15[2] per cent, which is equivalent to about 4.3 per cent sea water. He gives the maximum salt concentration in which *Potamogeton foliosus* can survive as 0.3[2] per cent, or about 8.6 per cent sea water. He also reports *Ruppia maritima* growing in water with a 7.7[2] per cent salt content, and *Potamogeton pectinatus* in 3.5[2] per cent. These concentrations are equivalent to 220.0 and 100.0 per cent sea water, respectively. Chemical analyses of the water of these lakes, however, show only a relatively small proportion of sodium chloride as compared to the relative amount in sea water. For example, lake water with a salt content equal to the amount in sea water contained only 20.0 per cent in terms of sodium chloride. Osterhout (57) previously had grown *Ruppia maritima* for 150 days in sea water (containing 2.7 per cent total salts) and had concluded that sea water is a "physiologically balanced solution."

Since Brongniart (14), in 1834, first discovered chlorophyll in the epidermis of a submerged angiosperm, *Potamogeton lucens*, botanists have used aquatic seed plants as material for physiological study. Most of such studies, however, have been concerned with photosynthesis and the factors, such as light intensity and carbon dioxide supply, which limit the process. Blackman and Smith (12), Harder (30), James (35), Pantanelli (59), Reinke (67), and Willmot (101) may be cited among those who have presented reviews of the literature dealing with the subject of photosynthesis in submerged plants. Arber (4) presents an excellent review of the important physiological studies made on aquatic angiosperms previous to 1920. Only experimental studies that are concerned with the physiology of submerged seed plants in relation to their growth and development in nature, and that have a direct bearing upon the present problem, will be reviewed here.

The most conspicuous physiological and anatomical characteristics of aquatic angiosperms, as compared with land plants, are the presence of chlorophyll in the epidermis, the small amount of cuticle, and the reduction in vascular tissue, as described by Chrysler (19). The cell walls of submerged plants were found by Devaux (26) to offer no more hindrance

University and to members of the staff of the Boyce Thompson Institute for suggestions and criticisms and to Mr. W. E. Corey of New York City, who furnished the funds needed to carry on the investigation.

[2] Decimal points in Metcalf's figures have been shifted two places to the right in order to correct his percentage values to agree with the figures he gives for parts per million and to make them conform to popular usage.

to the direct passage of dissolved gases than if they were merely thin plates of water. SAVAGEAU (77) proved by plasmolysis experiments with entire leaves of *Potamogeton pectinatus* that even a waxy cuticle of a submerged plant is no obstacle to the entry of liquids into the leaf cells. Based upon such anatomical characteristics, rather than upon experimental evidence, it has been concluded, since the plant is able to absorb water over the entire surface, that there is no current of water from root to leaf in an aquatic plant, as in a land plant, and that the roots of submerged plants serve only as organs of attachment. Despite the fact that such conclusions have been, at least to a large extent, disproved by experimental evidence, HANNIG (29, p. 200) in 1912, finding greater osmotic concentrations by several atmospheres in the tissues of roots than of stems and leaves of land plants, speaks of discovering also the same differences in submerged plants, in which case he says, "kein Transpirationsstrom existiert." BROWN (15, 16) has been one of the latest writers to subscribe to the view that the roots of aquatic plants function only as organs of attachment. *Elodea canadensis*, he found, is not dependent on its roots for the absorption of nutrient salts. He explains the fact that plants rooted in good soil grow better than those anchored over the same substratum because of the anchorage of the former near a greater supply of carbon dioxide. He further concludes, from experiments with *Elodea* in nutrient solutions and in tap water containing added carbon dioxide, that under natural conditions variations in the amount of carbon dioxide in the water are more likely to affect the growth of *Elodea* than variations in the percentage of nutrient salts in solution. Another group of workers holds an opposite view from that of BROWN and others.

The more modern work on the subject of absorption in submerged angiosperms may be said to begin with the observations and experiments of SAVAGEAU (76, 77, 78). He argues that if one of the uses of the circulation of water in the plant is to supply nutritive substances, this ought to be relatively important in the case of submerged plants, because the water in which they live is often less rich in dissolved salts than that which circulates in the soil. He notes that species of *Potamogeton* and *Naias*, among others, have well developed roots and that the root hairs persist longer than the other cells of the piliferous layer. Species of *Potamogeton* he found of two sorts, submerged without stomata and exposed with stomata. He states that the total surface of the floating leaves is always less than that of the submerged. His theory is that the processes of absorption, conduction, and giving off water necessitated by the floating leaves are not suddenly initiated at the moment the floating leaves reach the surface, but must have been in operation during the period when the floating leaves were still undeveloped, and likewise in those plants entirely submerged, since their roots serve not only mechanically for attachment but also for absorption. In his experiments, however, he used detached branches of sub-

merged plants, in which the cut stem had been sealed with cocoa butter and all roots had been removed. Under these conditions he found the plants able to live and develop new buds, thus justifying to a certain extent the current view that water was absorbed through the surface of the stems and leaves of submerged plants. He then performed a converse experiment in which he attempted to demonstrate, by direct measurement of the water passing through the stem of immersed cuttings, that under normal conditions aquatic plants absorb and give off water by a process comparable to that of land plants. He states that if the plants had been provided with roots the absorption would have been greater.

Hochreutiner (33), in 1896, challenged a statement made ten years earlier by Schenck in his "Vergleichende Anatomie der submersen Gewächse" that submerged plants absorb, through their leaves directly from the surrounding water, salts and carbon dioxide, and that a vascular system and stomata in such plants serve no purpose. The presence of a cuticle and well developed root hairs in many hydrophytes rendered the idea of absorption only through the leaves untenable to him. For verifying or denying of Schenck's assertion, Hochreutiner states there were open to him two methods: the use of nutrient solutions, or the use of dyes. Because of practical difficulties and possibility of numerous errors in the use of nutrient solutions, the dyes were selected. His experiment with *Potamogeton pectinatus* L. illustrates the method employed. Two vessels standing adjacent were used, one containing aqueous eosin solution and the other pure water. One cutting had its base immersed in the eosin to a length of 2 cm. and its upper portion in pure water. A second cutting had 9 cm. of its upper part in eosin and its base in pure water. The exposed parts were greased to prevent capillarity and the preparation was kept in a saturated atmosphere. After two days it was found that eosin could be detected in the main stem of the first plant, which was itself 20 cm. long, 15 cm. from its base and 16 cm. in the lateral branches. The second plant, having its upper part in eosin, showed a coloration only in the epidermis, the vascular tissue of the leaves being unaffected. Practically the same results were obtained with *Potamogeton crispus*, *P. densus*, and *Ranunculus aquatilis*. He concludes that in these plants there is an upward current of water; that there may be some absorption by the leaves, but it is slight compared to the absorption by roots; and that these aquatics obtain water and salts in a manner comparable to that of land plants.

Pfeffer (60, p. 242) expresses the opinion that a circulation of water in submerged plants is possible. He reviews the literature briefly and states that the experiments of Hochreutiner are not conclusive and that the opinion of Savageau is not supported by experimental evidence.

Some experiments, similar in principle to those of Hochreutiner, were performed in 1909 by Thoday and Sykes (86) with *Potamogeton lucens*

plants growing in the River Cam in July and August. They attached a small bulb of eosin to the cut end of a submerged branch and found a rise of the dye in the stem at a rate greater than 9 cm. per minute. This rise was found to be dependent upon the leaves, for the removal of all leaves and the apical bud retarded the upward current to a rate of 3 cm. in 32 minutes. When some of the leaves were removed the rate of transmission of the eosin was diminished roughly in proportion to the number removed. These writers term the upward movement of water in submerged plants "the transpiration stream." Barnes (7) in a review of their work says: "To call such a stream 'the transpiration stream' is manifestly absurd, unless one changes the meaning of the word transpiration." Barnes suggests, however, that the heating of the leaves may create the conditions necessary for the circulation of water in submerged plants. Arber (4), in her important book on water plants, uses the term transpiration instead of guttation, suggested by Burgerstein (17, 18) as more appropriate for submerged plants, because guttation is considered by her too awkward and ugly to be readily admitted into the English language.

Apparently, the first direct experimental evidence that roots of submerged plants serve not only for purposes of anchorage but also as organs for absorption was offered by Pond (63) in 1905. As experimental material he employed rooted plants of *Vallisneria spiralis*, *Elodea canadensis*, *Myriophyllum spicatum*, *Potamogeton obtusifolius*, *Potamogeton perfoliatus*, and *Ranunculus aquatilis* var. *trichophyllus*. In one experiment, roots of a plant were immersed in a bottle containing a solution of lithium nitrate (1 per cent in tap water) and separated from the surrounding tap water by means of a plug of cotton wool and vaseline. After 24 hours the upper parts of the plant were tested for lithium with flame and spectroscope. Lithium was found in all parts of the stem and leaves except the terminal node and leaf. None was detected in a root remaining outside the bottle, not even within 2 mm. of its union with the main stem. In another experiment lithium was found to travel upwards in the stem at a rate of 17 cm. in five hours. He concludes that mere diffusion will not account for these results, because, if the process were simply that, downward diffusion of the lithium salt would be more rapid and the root outside the bottle should show at least a trace of it. In a similarly arranged experiment, he succeeded in actually measuring the water absorbed by a single root of *Ranunculus aquatilis* var. *trichophyllus*. The root, 14 cm. long and well provided with root hairs, was found to absorb 5 cc. of water in 25 hours. From these experiments he concludes that roots of submerged plants are organs of absorption as well as of attachment, and that there is an upward current of water from roots to stem and leaves.

Pond also carried out a number of experiments for the purpose of determining the influence of the substratum and nutrient solutions on the

growth of several species of submerged plants. The plants were grown rooted in soil, rooted in washed sand, anchored over soil, and anchored over sand. Crystallizing dishes received the roots that developed from the anchored plants and prevented their contact with the substratum. He found, throughout, that the rooted plants grew much better than those anchored over soil. He also tried growing the plants in Sachs' nutrient solution, but found this the least favorable of the five conditions, as several of the plants died. Plants grew better in the nutrient solution and soil than in the nutrient solution and sand. There was no root development in the nutrient solution without a substratum, and only a few roots developed in the nutrient solution and sand. There was, however, an abundant root development in control cultures in tap water. Pond deduced that the primary cause of retarded growth in the case of the plants anchored over a substratum was their inability to obtain sufficient amounts of phosphorus and potassium and possibly other elements. In some cases, he found that such plants were not only stunted in growth, but had their tissues gorged with an abnormal amount of starch. He concludes that protein synthesis is inhibited by an insufficiency of phosphorus and potassium, and that pathological conditions then arise which permit the accumulation of starch. He states that Sachs' nutrient solution contains ingredients in unsuitable proportions for aquatic plants, but if this nutrient solution is injurious the soil and sand particles must partially neutralize its effect. From these experiments he concludes that the six species of submerged angiosperms studied by him are dependent upon their rooting in the soil for optimum growth and that they cannot survive a single season if denied a substratum of soil.

Results similar to those of Pond were obtained three years later by Snell (83), whose experiments may be illustrated by a single example. Ten terminal cuttings of *Elodea canadensis*, 10 cm. in length, without roots or lateral branches, were planted under water in soil in which they were allowed to take root. Another set of ten cuttings, approximately equal in size, was placed in the same glass vessel, but the cuttings were supported above the substratum in such a way that their roots were unable to come in contact with the soil. All the cultures were rotated in order to provide uniform lighting. After four weeks the cuttings were measured. It was found that the cuttings rooted in soil had grown much more rapidly, their total length amounting to 308.0 cm. as compared with 177.5 cm., 114.0 cm., and 114.0 cm. for those anchored over soil, rooted in quartz sand, and anchored over quartz sand, respectively. Only roots developing in a substratum showed root hairs. These results in general confirm those of Pond except that plants anchored over soil grew better than those rooted in sand.

Snell also carried out some experiments to determine the influence of

nutrient solutions on the growth of *Elodea canadensis*, *Potamogeton densus*, and *Ranunculus fluitans*. No success was obtained with cultures in 0.5, 1.0, 1.5, 2.5, 5.0, and 10.0 per cent Crone's nutrient solution, as decay and cessation of growth soon set in. At first the injurious effect of the nutrient solution was attributed to the toxicity of the distilled water with which the solutions were made up. In solutions made up with tap water, however, little growth occurred, and the plants soon decayed. When the experiments were renewed with nutrient solutions made up with tap water and carried out at a temperature of 12° C., much better results were obtained, but even then growth in the nutrient solutions showed no great improvement over that in the tap water controls.

In spite of numerous experimental attempts, Snell could not detect the absorption of lithium nitrate by *Elodea canadensis* and *Ranunculus trichophyllus*, as Pond had done. He always found a red line in the spectrum but he believed this to be the calcium line, caused by the natural deposition of calcium carbonate on the plant leaves. Positive results, however, were obtained with potassium ferrocyanide solution which formed Berlin Blue in the conducting vessels with ferric chloride. In one case the roots were found to absorb the solution which in four days reached the tip of the plant, a distance of about 50 cm.

In additional experiments, in which absorption through the roots or stems and leaves was controlled either by a mechanical arrangement or by removing the roots and cementing the wounds with plaster of Paris and a low melting paraffin, absorption was found by Snell to be much greater through the roots than through the stems and leaves. He concludes that the roots of such aquatic plants as *Elodea canadensis*, *Potamogeton densus*, *Ranunculus fluitans*, and *Sagittaria natans* serve not merely as anchorage organs but for the absorption of nutrient salts; that under normal conditions there is in water plants an ascending current of water to the growing points; that the epidermis of the stem and leaves is permeable to solutions; and that, under conditions of absorption, nutrients can be taken into a submerged plant over its entire surface. *Pistia stratiotes*, a floating plant, was found, however, to absorb nutrients only through the roots and through the underside of very young leaves. The roots of *Lemna minor*, another floating plant, were found to have only a mechanical importance, as absorption occurs only through the underside of the leaves, and the roots serve only to check the moving about of the plant by the motion of the water.

The preceding review of literature shows that the experiments and opinions of botanists on the function of roots in submerged plants, and on the influence of substratum and nutrient salts on aquatic plant growth, are by no means in agreement. For example, the results of Pond (63) and Snell (83), bearing on these questions, seem impossible to reconcile with

the more recently published results of Brown (16), who finds that the difference in growth between rooted plants and those merely anchored over a substratum can be altogether eliminated by passing free carbon dioxide through the water several times a day. He considers that the non-rooted plants do not suffer from a lack of nutrient salts, but chiefly from a lack of the supply of carbon dioxide which is given off from soil containing decomposing organic matter. Commenting on this difference of opinion between Pond and Brown, Arber (4, p. 266) says: "The divergence of these workers' views indicates a direction in which further work of a critical nature is markedly needed." While a deficiency of carbon dioxide is found to be an important factor limiting the growth of aquatic plants, quantitative results of the writer's experiments show that a constant supply of this gas does not eliminate the difference in growth between plants rooted in soil and those merely anchored in solutions. The rooted condition is found in general to be an expression of the degree of vigor of aquatic plant growth, and its value to differ somewhat with the species. Growth is shown to be stimulated by the addition of dilute nutrient solutions to the medium. These results differ appreciably from those previously reported by Brown (16), and are not in entire agreement with those of Pond (63). Additional quantitative results show the influence of various dilutions of sea water on the growth of submerged plants, and the light requirements of one species, *Potamogeton pectinatus* L.

DESCRIPTION OF THE REGION

The upper part of Currituck Sound in North Carolina and the connected waters of Back Bay and North Bay in Virginia (Figs. 1 and 5) are situated approximately between 36° 15′ and 36° 45′ north latitude and between 75° 50′ and 76° 05′ west longitude. These waters, covering a surface of nearly 300 square miles or 200,000 acres, are the northern section of a large area of inland waters and empty southward into Albemarle Sound. From these the Atlantic Ocean is walled off by a long, narrow, sandy, barrier reef which averages less than a mile wide. This reef is almost a linear chain of sand dunes, some of which according to Clark and Miller (20), Sanford (75), and Stephenson and Johnson (84) range 70 to 90 feet in height. In several places, however, the dunes are separated by strips of flat beach, ranging in width from a few hundred feet to one mile. These flats usually do not exceed 10 feet in elevation above mean sea level and permit sea water in time of severe storms to flow into the inland waters. A few of these low places were formerly inlets from the sea, the last of which Jewett (36) states closed about 1835. Cobb (24) expresses the opinion that this beach is subsiding, but Clark and Miller (20) seem to indicate that it is increasing in size and eventually, with the gradual filling in of the coastal lagoons, will be connected with the mainland. The main-

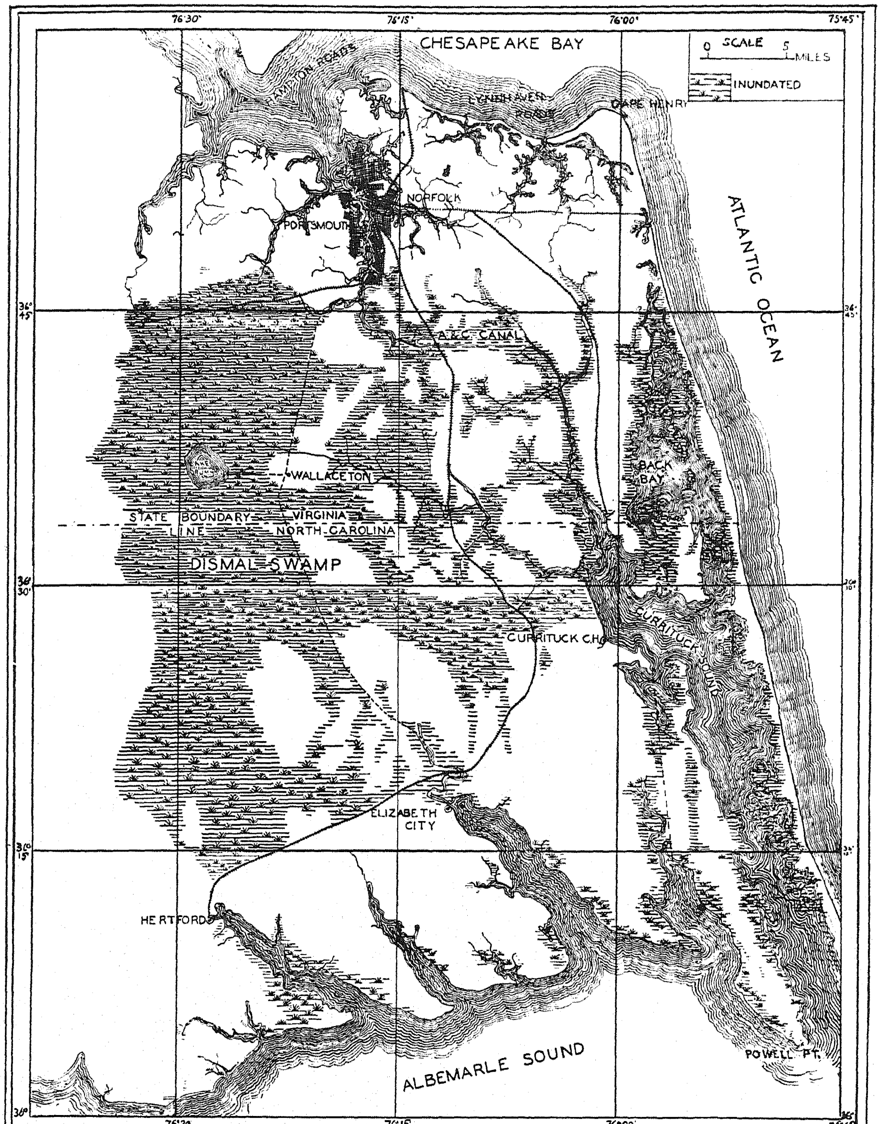

FIGURE 1. The Back Bay, Virginia, and Currituck Sound, North Carolina, region, showing the relation of these waters to the Albemarle and Chesapeake Canal, Norfolk Harbor, and Hampton Roads.

land on the west is low and sandy, with no elevation, according to Clark and Miller (20), Sanford (75), and Stephenson and Johnson (84), exceeding 25 feet above mean sea level. These writers refer to the entire surface of Princess Anne County, Virginia, and Currituck County, North Carolina, in which the waters under consideration are situated, as being composed of the Talbot and Pamlico formations of the Columbia group of the Pleistocene series.

Currituck Sound is fed from the north and west by numerous creeks and by three rivers, North Landing, Blackwater, and Northwest, which have their origin in or near Dismal Swamp. Only a few small creeks flow into North Bay and Back Bay, the waters of which are connected with Currituck Sound by a narrow channel winding through marshy meadows. The slope of the shores is usually very gentle, and almost the full extent of the shore lines, even those of the creeks and rivers is fringed by marshes of varying width. Along the creeks and rivers the marsh consists ordinarily of a narrow strip on each side of the channel, while bordering the larger bodies of water it sometimes takes the form of a meadow of considerable width; for example, the Great Marsh, between Currituck Sound and Back Bay is more than 10 square miles in area. Except for narrow, shallow channels, marshes separate Back Bay from Currituck Sound and Currituck Sound itself almost in half. The dominant plants of these marshes are *Spartina glabra* Muhl. and *Spartina cynosuroides* (L.) Roth. in the firmer parts and *Typha angustifolia* L. in soft patches and in the borders of the marsh ponds.

The water of all the creeks and rivers is dark brown in color, because of its content of finely divided humic material, and is usually slightly acid in reaction, especially the sherry-colored water coming from the juniper (*Chamaecyparis thyoides* (L.) B. S. P.) and cypress (*Taxodium distichum* (L.) Richard.) sections of Dismal Swamp. The water of Currituck Sound and the bays to the north, however, is normally clear and transparent and nearly neutral in reaction. While McAtee (51) and Johnson (37), residents and sportsmen who have visited the region continuously for a great number of years, have stated that the water of Currituck Sound and Back Bay was clear, fresh, and potable before the year 1918, there has been no record published, so far as is known, of the salt content of this water prior to the beginning of the present investigation in 1925. It is now brackish and very turbid. Its average depth is about 5 to 6 feet, and maximum depths do not exceed 10 feet except in the channels, some of which have been dredged. Many large coves are no more than 2 feet in depth, and are often so shallow that strong winds stir the mud from the bottoms. There are no regular tides in these waters, such as one might expect to occur because of their proximity to the sea, but occasionally there is a period of higher water following excessive rainfall or a strong, protracted,

south wind, which backs up the water from the lower Sound, or a period of lower water after a north wind of more than 24 hours duration.

The bottoms of these inland waters are covered for the most part with a layer of black mud, varying in thickness from a few inches in the deeper water to two feet or more in the shallow bays and coves. This mud is composed mainly of partially decomposed vegetation, but is sometimes found mixed with very thin strata of white sand which has been carried by east winds from the beach dunes. There is, however, very little sandy bottom in any of these waters except in a few places along the shore of the mainland or the higher islands. Beneath the mud and often outcropping in the channels and deeper water is a soft blue clay, frequently containing numerous, sometimes entire, beds of large mollusk shells. In a few places these shell beds extend to the surface of the water, and are said by Stephenson and Johnson (84) to be of Pleistocene origin.

The climate of the region is characterized by a long growing period, mild winters, slight daily variations in temperature, abundant sunshine, and a heavy and well distributed rainfall. The following figures, taken or compiled from the U. S. Weather Bureau (95) records, for the Norfolk station are considered applicable to the region, since the farthest point is within a 50-mile radius of Norfolk. The figures represent normal averages. The mean annual temperature is 15.2° C., the daily range of temperature 8.8° C., the annual percentage of possible sunshine 51, the annual humidity in percentage of saturation 71, the annual rainfall 106.6 cm., the number of rainy days per annum 131.3, and the average maximum wind velocity 15.0 kilometers per hour. The normal number of days per annum with a temperature above 6.0° C. is 295. The absolute maximum temperature is given as 39.0° C. and the minimum as −16.6° C.

The normal wind direction in midsummer is almost exactly opposite to the normal direction in midwinter. In January the prevailing winds at Norfolk are from slightly west of north. In July, on the other hand, the direction is somewhat west of south. As will be pointed out later, the direction, the velocity, and the duration of the winds in recent years have been an important factor influencing the plant life of Currituck Sound and Back Bay.

There has been little information published concerning the aquatic flora of the region. Kearney (38) in 1898 made a botanical survey of Dismal Swamp and adjacent parts of Virginia and North Carolina, but he does not mention the plant life of Currituck Sound and Back Bay. His report indicates that he did not visit these inland waters, as he states that he travelled only to a point along the coast eight miles south of Virginia Beach, which would not have brought him to Back Bay. McAtee (48) visited the region in 1909 and published some notes on the flora of Church's Island in Currituck Sound and included therein some aquatic plants of the

Sound. He lists *Potamogeton pectinatus* L. as being the dominant plant of the water and extremely abundant; *Naias flexilis* (Willd.) Rostk. & Schmidt, as abundant; *Vallisneria spiralis* L., as abundant, especially along channels, in places over soft muddy bottom, and in creeks in the marshes; *Potamogeton perfoliatus* L., as common; *Ruppia maritima* L., as scattered; and *Potamogeton foliosus* Raf., as scarce in the Sound, but common in ponds and streams of the marshes. Johnson (37, p. 1) in 1929, writing on the conditions of these waters says:

"These fresh waters of upper Currituck Sound and North and Back Bays, covering an area of nearly 300 square miles or 200,000 acres, are all shallow, varying from two to seven feet in depth. The bottoms are a rich marl, and over the entire area there formerly flourished a luxurious growth of fresh water aquatic plants including several varieties of pond weed and wild celery, which, in leaf, stem, and tuber, was attractive, nutritious, and palatable to wild fowl. These heavy growths with their spreading roots protected the bottoms from wind disturbance to such an extent that the waters were clear and afforded an ideal feeding ground for black bass and perch; the waters were so fresh and pure that hunters and fishermen found them palatable for drinking purposes."

Furthermore, he quotes a letter (37, p. 16), dated "Nov. 14, 1929," from Arthur M. Hyde, Secretary of the U. S. Department of Agriculture, to Wesley L. Jones, Chairman of the Committee on Commerce of the United States Senate, which states in part:

"The Biological Survey has had representatives studying the feeding conditions for wild fowl in the Currituck Sound region on numerous occasions dated back to 1909. At that time water in Currituck Sound was so clear that the grains of sand on the bottom could almost be counted and was so pure in quality that no one thought of taking a supply of drinking water with them for excursions on the Sound but dipped up water for a drink wherever they happened to be. The stand of wild duck food plants was then unsurpassed in variety and quantity and Currituck Sound fully upheld its longstanding reputation as being one of the greatest resorts for wild fowl on the continent."

While inhabitants and sportsmen, who have visited the region for many years, have stated that the growth of submerged plants was formerly so thick during the summer months as to make it difficult to get a boat through the water, the references above, so far as can be determined, are the only published records dealing with the aquatic plants of Currituck Sound and Back Bay prior to the beginning of the present investigation.

ECOLOGICAL INVESTIGATIONS

PRELIMINARY OBSERVATIONS

Submerged plants. In 1926 a relatively small quantity of aquatic seed plants remained in the region. At that time the larger and deeper parts of Currituck Sound and Back Bay, constituting about two-thirds of the total area, were barren. The growth of submerged angiosperms was limited mainly to the waters of North Bay, the southern part of Currituck Sound, the shallow coves and margins of the larger bodies of water, and the ponds and ditches in the marshes. Plants had disappeared from the main and deeper parts of Back Bay and Currituck Sound. *Potamogeton pectinatus* L., as McAtee has stated, was the dominant aquatic angiosperm of the region, as it comprised at least 60 per cent of the total aquatic plant growth. Other plants in the order of abundance were: *Naias flexilis* (Willd.) Rostk. & Schmidt, *Vallisneria spiralis* L., *Potamogeton perfoliatus* L., *Ruppia martima* L., *Ceratophyllum demersum* L., and *Potamogeton foliosus* Raf. This list represents the aquatic angiosperms occurring in such quantity as to be of any economic importance to the region. Many others, such as *Lemna minor* L. and *Elodea canadensis* Michx. were observed in sloughs along the edge of the marshes, but their numbers were too few to be of any practical importance or to require study. In addition to the seed plants mentioned, *Chara* spp. and *Nitella* spp. occurred in appreciable quantities in North Bay and along a few sandy shores, but the quantity of *Chara* then present must have represented only a small proportion of that reported as occurring in 1909 by McAtee (49, p. 63), who stated that it blanketed "the bottom of almost the whole of Currituck Sound."

There was very little zonation of plants to be observed. In waters supporting plant growths, *Potamogeton pectinatus* invariably occupied the deeper parts, but it did not always occur in pure stands, being frequently mixed on soft muddy bottoms with *Vallisneria spiralis*, *Potamogeton perfoliatus*, and sometimes *Ruppia maritima*. The shallow waters, depths of two feet or less, were inhabited by pure or mixed stands of *Naias flexilis*, *Vallisneria spiralis*, and *Ruppia maritima*. In depths from two to four feet growths of all seemed to blend together. At greater depths *Potamogeton pectinatus* usually occurred alone. This fact may be related to the particular type of growth of the plant. It is propagated in nature both from seed and vegetatively from starchy tubers. Plants originating from tubers have been observed to reach the surface of the water, from a depth of six feet, early in May. The plant stem then is unbranched, but soon thereafter profuse branching takes place along the stem near the surface of the water. Relatively few plants can, therefore, cover a considerable area and crowd out plants such as *Vallisneria spiralis*, which in this region makes little growth before June. *Naias flexilis* was the dominant plant in the shallow waters, as it occurred in pure stands in many ponds and over almost the

whole of North Bay, but it was often found mixed with *Vallisneria spiralis* and *Potamogeton perfoliatus*. Most of the small creeks and ditches were completely filled with *Ceratophyllum demersum*, which seemed to be able to thrive in the dark brown water draining from the swamps. Many small ponds, especially those receiving drainage directly from the mainland, were inhabited only by *Potamogeton foliosus*.

During the latter part of April, 1926, *Potamogeton pectinatus* plants growing near the channels and the margins of the barren areas were observed to be covered by growths of the brackish-water hydroid, *Cordylophora lacustris* Allman. At first these growths were composed of single, profusely branched colonies extending from the base of the plants along the stem towards the tip, but not to the parts of the plants lying flat upon the water surface. After a month, however, there were usually so many colonies entwining the plant stems as to cause them to appear often more than a centimeter in diameter. By the first of June the hydroids had spread to almost all the *Potamogeton pectinatus* plants growing in the main bodies of water, and because of the production of enormous quantities of gonophores in the hydroid colonies plants in the clearer water appeared pink rather than green in color. At this time of the year other species of submerged aquatics mentioned had made little growth and were scarcely affected by hydroids. Growth of these animals, however, was not limited to that occurring on *Potamogeton pectinatus*, but was to be found on leaves of sedges drooping into the water, on pilings, channel stakes, fishing seines (set-nets), shells, and even on the bottoms of motor boats. There seemed to be a gelatinous secretion from the colonies which enabled them to adhere to a smooth flat surface, such as the bottom of a boat.

For several weeks during midsummer no living hydroids were to be found on the plants. Death of the colonies occurred in early June as soon as the young embryos formed and dropped to the bottom. This left upon the plants a decaying mass of gelatinous material which was known locally as "slur" and which was believed by the inhabitants of the region to be one of the principal causes for the disappearance of the duck-food plants from the waters. A similar but much lesser growth of hydroids occurred again in late August and in September. Figure 2 shows a typical late growth of the hydroid on several species of plants. Figure 3 shows the manner in which a hydroid colony entwines a plant.

Allman (1) first described *Cordylophora lacustris* in 1853 and indicated that it was a fresh-water form. Schulze (80), however, in a monograph on the structure and development of the animal published in 1871, stated that it reached its maximum development in brackish water. Ward and Whipple (98) report *Cordylophora lacustris* as both a fresh-water and brackish-water form, but state that the most favorable conditions for it are in brackish water and that there it attains most luxuriant develop-

ment, the colonies being more stalwart and the ascending branches more than twice as large as in colonies from fresh water. Garman (27) reported its occurrence in a Kentucky creek in 1922, but said nothing concerning its abundance. Smith (82) in announcing its presence in the Illinois River in 1910 suggested that it may have been introduced into the Chicago re-

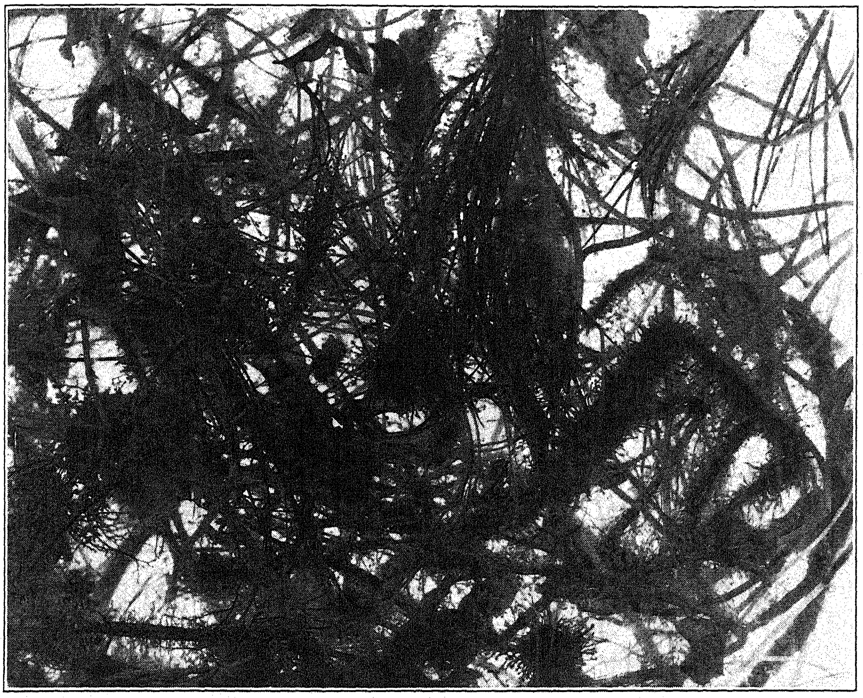

FIGURE 2. Submerged plants covered with colonial growths of the brackish-water hydroid, *Cordylophora lacustris* Allman.

gion by vessels from the Atlantic coast and then subsequently to have been carried through the Chicago Drainage Canal into the Illinois River. He speaks of the animal (p. 67) as "a species of hydroid commonly found in brackish water and less frequently in fresh water." Richardson (71) listed a single occurrence of the animal in the Illinois River at Kingston in 1923. While Hargitt (31, 32) described this hydroid as a form frequenting both fresh and brackish water, he informed the writer that he had never seen before such thrifty specimens of the animal as those sent to him in 1926 from the brackish water of Currituck Sound. Lenz (44), in 1928, listed it more specifically as a mesohyaline organism, inhabiting water containing a chloride content equal to approximately 3 to 30 per

cent sea water. From these accounts it would seem that this hydroid is more strictly a brackish-water organism. How long it has existed in the Currituck Sound region is unknown, but according to inhabitants it became noticeable, as "slur," about 1922 when the water had become decidedly brackish and the duck-food plants had begun to die. It was rarely found during the course of this investigation in the fresh waters of the region.

FIGURE 3. Photomicrograph (about 50×) of *Cordylophora lacustris* Allman entwining a leaf of *Potamogeton pectinatus* L.

This hydroid is strictly a plankton feeder, and is thus a competitor of the fishes. Its abundance in the region may be correlated with the scarcity of fish, as Jewett (36) quotes evidence that the fresh fish catch in Currituck Sound dropped from 2,000,000 pounds in 1920 to 300,000 pounds in 1927, and the latter catch comprised mostly bottom-feeding forms.

Cordylophora lacustris is not parasitic upon the submerged seed plants. While such growths as those shown in Figure 2 undoubtedly suffocate portions of the plants and the twining colonies (Fig. 3) injure the stems mechanically, the principal damage seems to come from the gelatinous material left upon the plants after zoophytic growth has ceased. During the life of the colonies this material is slippery to the touch, but after death it is more like sandpaper, because of the great amount of sediment and organisms that collect in it. It then apparently becomes a favorable

medium for the growth of various organisms. Just as Schrenk (79) reported their growth in the mucilaginous substance on *Brasenia peltata* Pursh., bacteria and diatoms seemed to thrive in this gelatinous material. Often the diatoms collected in such numbers as to make the plant stems as brittle

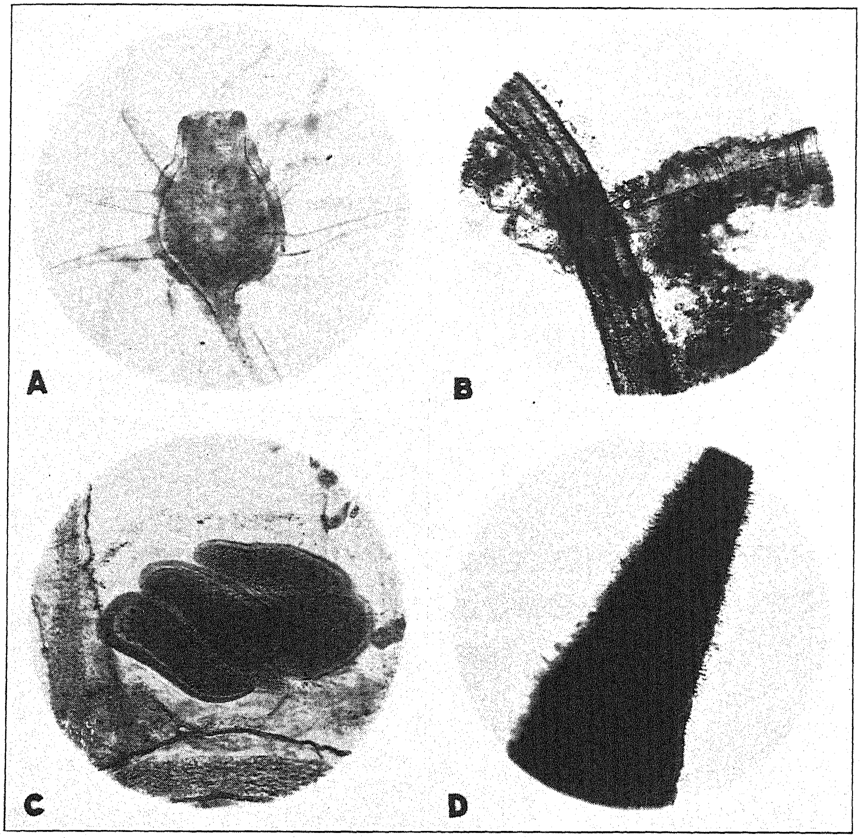

FIGURE 4. Photomicrographs (about 50×) of *Cordylophora lacustris* Allman. (A) A typical hydranth; (B) A stalk of a colony with its gelatinous secretions; (C) Embryos; (D) Diatoms growing in the gelatinous secretions left by hydroid colonies on a leaf of an aquatic plant.

as thin glass. Larvae, worms, rotifers, protozoans, and fungi were usually found in great numbers in this material. Among the latter, Bourn and Jenkins (13) identified a strain of *Rhizoctonia solani* Kühn and found by experiments that it was parasitic upon aquatic plants. Dark lesions usually appeared at places of contact between the hydroid colony and the plant stem, and the fungus was isolated from margins of these lesions.

Whether injured by hydroids or killed by fungi and other organisms, the plants, with entirely or partially decayed stems, became detached and were washed ashore during the summer, and later decayed in large windrows. While *Rhizoctonia* was found sometimes to attack plants not infested with hydroids, nearly every dead plant in the region was observed to have clinging to it either hydroid colonies or their remains.

The twining method of a hydroid colony is illustrated in Figure 3. In Figure 4 there are shown: (A) a feeding hydranth; (B) a portion of a colony, with its gelatinous secretion ("slur"); (C) young embryos, which give to the colony a pinkish hue; and (D) diatoms adhering to an aquatic plant after having been covered by hydroid secretions.

Water. One of the most noticeable characteristics of the water of the region was its extreme turbidity, especially when the wind velocity was moderate or greater. The water was dark gray in color and was never observed to be transparent except in the few places where plant growths were well established. In such places the water was clear and transparent, quite unlike the dark brown water of all the tributaries. The main bodies of water, however, were turbid during the entire course of this investigation. In the shallow parts of Back Bay, where the bottoms were composed of a thick layer of soft mud, it was usually difficult to determine where the water left off and the bottom began, and one preliminary test showed that over 90 per cent of the sunlight was absorbed by the first inch of water.

The water, especially in the shallow regions, ordinarily had a foul odor because of the great amount of decaying organic matter contained in it. Gases were given off continuously from the soft muddy bottoms, and appreciable volumes arose to the surface when the bottom was disturbed with a boat oar. The identity of these gases was not determined. While the odor was similar to hydrogen sulphide, there are organic substances, commonly found in sewage polluted water, known to have about the same odor.

Large globules of oil and oil films were found frequently on the surface of Currituck Sound, and when the mud on the bottom was disturbed an oil film invariably arose. No oil, however, was observed in the waters of Back Bay.

Another characteristic of the water was its brackishness. It formerly was supposed to be fresh, and the change from fresh to brackish water was claimed by sportsmen and inhabitants of the region to be one of the principal causes for the disappearance of the duck-food plants from the waters.

The conditions of the water as described would seem to place these inland waters, according to Kolkwitz and Marsson's (41, 42) classification or ecological system, in the alpha-mesosaprobic zone of existence, if not in the polysaprobic. The black sludge deposits accumulating on the bot-

tom, the presence of flagellate and ciliate protozoa, and the absence of fish suggest the polysaprobic. These writers, however, list *Cordylophora lacustris* as an oligosaprobic organism. Yet this animal thrives abundantly in the foul waters of Currituck Sound. In addition to great numbers of bacteriverous protozoa (colorless flagellates and ciliates), red sludge worms (*Tubifex*) occurred in abundance. Richardson (69, 70, 71) regarded tubifex worms as indicative and tolerant of a high degree of water pollution.

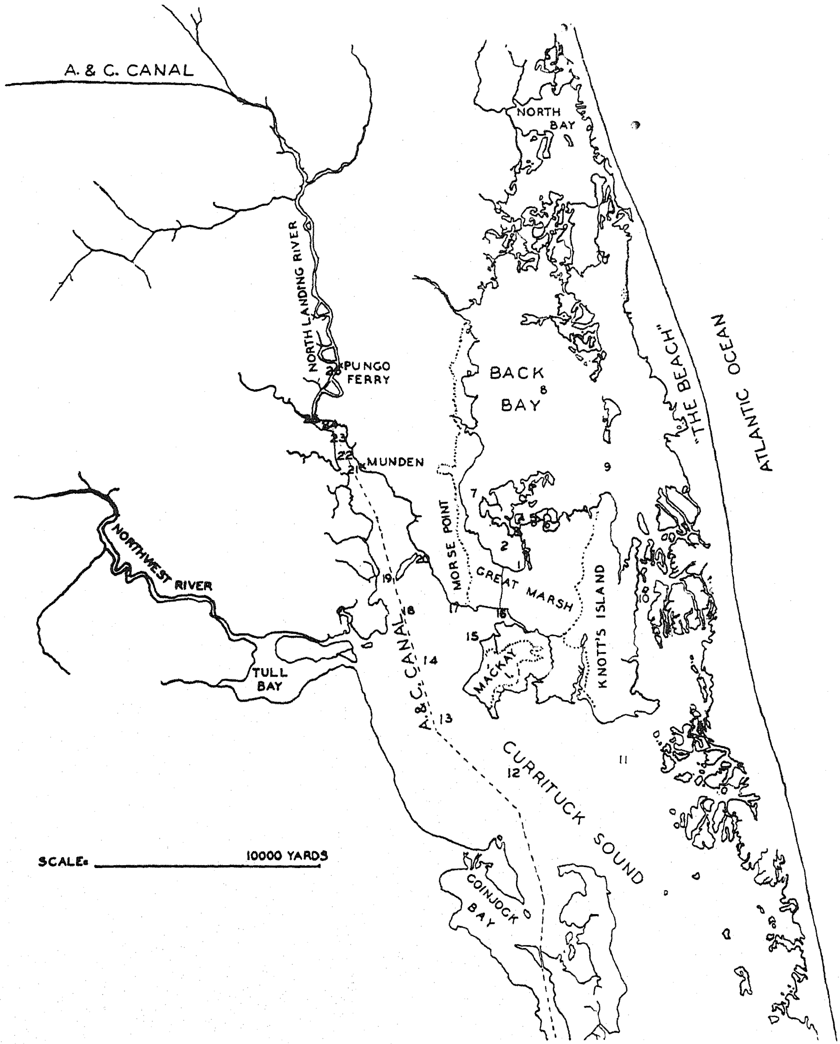

FIGURE 5. Map of the Back Bay, Virginia, and Currituck Sound, North Carolina, region, showing the location of salt testing stations, named and listed according to their order in Figure 6.

Changes in natural conditions. The construction of the Albemarle and Chesapeake Canal has been the only major change in the natural conditions of the region. This is a sea level canal, connecting Chesapeake Bay at Norfolk with the waters of upper Currituck Sound, was completed about the year 1860 and was operated until 1912 by a private company. From its completion the canal contained a tidal guard lock at its northern end for the purpose of equalizing differences in water level caused by lunar tides influencing the water at the northern end, Currituck Sound being tideless, and to prevent salt water flowing through the canal into Currituck Sound. The U. S. Government purchased the canal in 1912 and made it a part of the Intracoastal Canal System. The tidal guard lock was removed in 1918 in order to facilitate navigation during the World War and to permit the enlargement and deepening of the canal. Before the completion of these improvements in 1922 complaints were made to the U. S. Government that salt water flowing through the canal into Currituck Sound was damaging plant and fish life. In a resultant investigation Jewett (36) of the U. S. Army Engineers reported that currents were observed to flow southward through the canal continuously for as long as 22 hours and 40 minutes, regardless of the rise and fall of lunar tide at Norfolk.

All the sewage from the cities of Norfolk and Portsmouth, all the industrial wastes from the great industrial plants and shipyards situated in these cities and around Hampton Roads is dumped directly into the water, and the sewage and wastes from Norfolk and Portsmouth empty almost in the very mouth of the Albemarle and Chesapeake Canal. Jewett (36) recorded velocities of the southward currents through the canal as high as 2 miles per hour. With such a velocity and with lengthy southward flows as reported a great amount of waste material must be carried into Currituck Sound. It has been observed that parts of Currituck Sound filled up two inches in a single season.

ECOLOGICAL METHODS

Salinity. In order to determine the salt content of the water, its variations, and its sources, monthly analyses were made regularly at each of the 26 stations numbered and located in Figure 5, and listed in order in Figure 6. These stations begin in Back Bay and extend, in the order given, through Back Bay, Knott's Island Channel (connecting Back Bay and Currituck Sound), Currituck Sound, North Landing River, and the Albemarle and Chespeake Canal to Pungo Ferry, a distance of over 30 miles. The analyses at all stations were made on the same day during the last week of each month. For these tests the method described by Denny (25) for total chlorides by titration with silver nitrate was used, as this method has been found, by checking with gravimetric methods, to be sufficiently accurate, rapid, and well suited to field work.

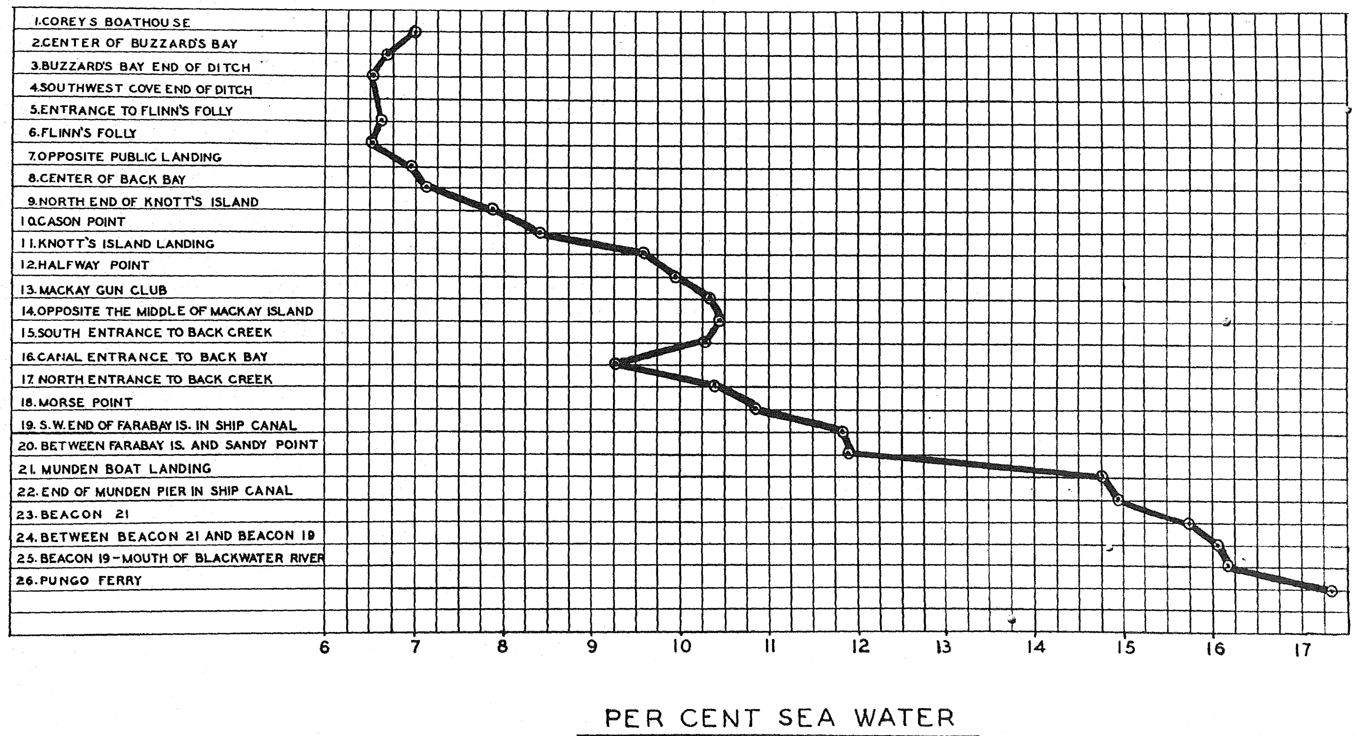

FIGURE 6. The average salt content of the waters of Back Bay, Virginia, and Currituck Sound, North Carolina, for 48 months. The averages are higher in the northern part of Currituck Sound and in the Albemarle and Chesapeake Canal at Pungo Ferry. Dips in the curve are because of the close proximity of some stations to the mouths of fresh streams.

Determinations were also made of the salt content of the bottom muds, the soils of the marshes, and various well waters of the region. Tests were made of the salt content of the water seeping into holes dug in low places along the barrier beach to determine the possibility of seepage of ocean water into the inland waters.

Light. For the purpose of measuring the penetration of total solar energy into the water a modification of the Shirley thermoelectric radiometer was used. The apparatus differed from that described by Shirley (81) in that it was somewhat larger, contained a supporting element in the neck of the flask, the thermopile was a little more sensitive type, and the flask was sealed and made water-tight with a rubber stopper.

On every clear day during the seasons of 1929 and 1930 readings were taken with the radiometer in the centers of North Bay, Back Bay, and Currituck Sound. These were made at one-foot levels from the surface to the bottom just after midday when the sun was approximately overhead. The directions for the operation of the apparatus given by Shirley were followed throughout.

Dissolved oxygen. Since the physical appearance and the odor are, at best, only coarse measures of existing conditions of pollution or purity of waters, a method capable of giving more refined results was sought. A review of the literature dealing with studies on stream pollution revealed the fact that the dissolved oxygen content of the water was considered the best index of the degree of pollution or of self-purification of waters. Richardson (69, 70, 71), Thompson (87, 88), and Wiebe (100) in reports on their studies of polluted waters of the Mississippi basin emphasized the deficiency of dissolved oxygen and considered it a measure of the degree of pollution. Whipple (99) regarded the dissolved oxygen content in this connection as a sensitive, rational, and readily applicable yardstick.

For determining the dissolved oxygen content the Rideal Stewart modification of the Winkler method as outlined by the American Public Health Association (2) in its "Standard methods for the examination of water and sewage" was used. Samples of water at various depths were collected in standard depth-sampling bottles. Titrations were made immediately in the field. Determinations of the dissolved oxygen content were made throughout the region frequently during June, July, August, and September, 1930.

Carbon dioxide. Determinations of the content of dissolved carbon dioxide in the water were made according to the titration methods given by the American Public Health Association (2). These were made only occasionally during each season.

Hydrogen ion concentration. The extreme turbidity and the color of the water in the region did not permit the use of colorimetric methods for determining its hydrogen ion concentration. For this purpose the quin-

hydrone method was employed, but the results obtained were so constant that only occasional tests were made.

Temperature. Using a standard flexible-tube centigrade thermometer adapted to recording temperatures at depths of 15 feet and less, temperatures were taken at various depths at the time of making tests of the salinity, of the penetration of solar energy, and of the content of dissolved gases.

Rainfall and winds. For the purpose of correlation with the salinity of the water and the flow of currents through the Albemarle and Chesapeake Canal, records of the amount of rainfall, and the direction, velocity, and duration of the winds in the region were obtained monthly by correspondence direct from the Norfolk station of the U. S. Weather Bureau.

Experimental plantings. During the first three years of the investigation attempts were made to find varieties of the duck-food plants already mentioned that might withstand the conditions existing in Currituck Sound and Back Bay. Trials were made with the three most important duck-food plants inhabiting the region, *Potamogeton pectinatus*, *Potamogeton perfoliatus*, and *Vallisneria spiralis*. Specimens of these species, no less than 10,000 in each case, were obtained or collected from fresh streams and lakes in Minnesota, Wisconsin, Pennsylvania, Maryland, Louisiana, and Florida. Similar collections were obtained also from the brackish waters (5 to 25 per cent of sea water) of the Hudson River in New York and Barnegat Bay in New Jersey. In addition to entire plants, many thousand tubers of *Potamogeton pectinatus* and winter buds or rhizomes of *Potamogeton perfoliatus* and *Vallisneria spiralis* were obtained or collected from each of the localities mentioned. Furthermore, collections of entire plants, tubers, rhizomes, and seeds of all the duck-food plants native to the region were made throughout each season from the immediate locality, wherever the plants were found to grow, by a crew of from four to eight men with boats and small barges. Plantings were made in the open barren areas, in ponds and coves connected directly to the open water by ditches or creeks, and in ponds protected, at least partially, from the influence of the outside water by means of bulkheads. In the latter case, bulkheads were constructed across creeks or ditches leading to the ponds by driving double rows of heavy sheet piling and filling in the intervening space with soil. These bulkheads acted more as filters than to prevent water flowing into or out of the ponds. Figure 7 illustrates such a bulkhead more than 400 yards long extending across the mouth of a natural cove.

Five of the experimental ponds, each several acres in area, were natural ponds in the marshes. These were shallow, ranging in depth from a few inches on the margins to three feet or more in the center. The bottoms were composed of a thick layer of soft black mud, beneath which was blue clay. One experimental pond, 100 acres in area, was entirely artificial, hav-

ing been dredged in the marsh to a depth of six feet into a blue clay subsoil. Another 100-acre experimental pond was formed by constructing a bulkhead across the mouth of a natural cove (Fig. 7) and by deepening it to four feet with a dredging machine. The bottom of the dredged portion was then covered with a layer of marsh soil.

Various methods of planting were tried. Wherever the depth of water permitted, plants, tubers, and rhizomes were placed directly in the mud by hand. In water too deep for this method plants were put out in soil,

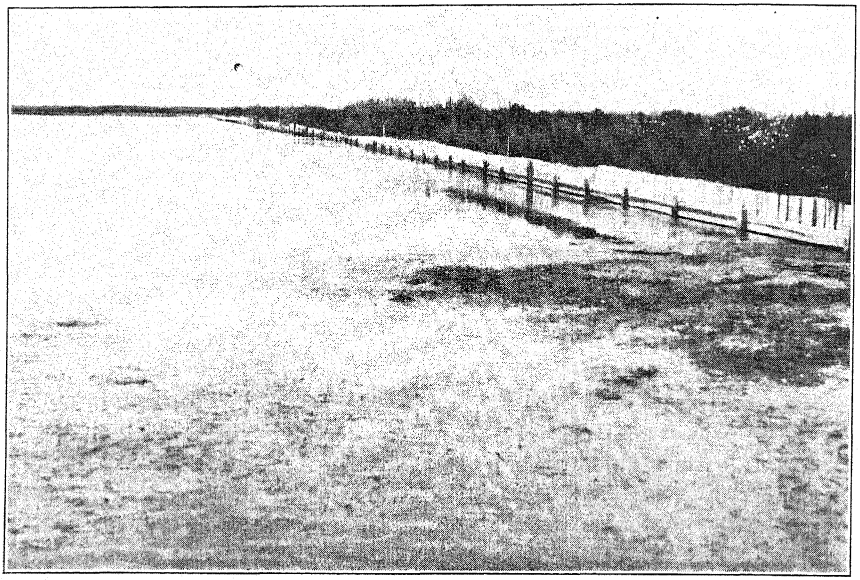

FIGURE 7. Aquatic angiosperms growing in a cove of Back Bay, protected from turbid, polluted water by the bulkhead on the right. The plants in the immediate foreground are growing in water four feet in depth.

often in the same soil in which they had been growing, contained in small fruit baskets and these sunk to the bottom. Other methods were to imbed tubers and rhizomes in balls made of stiff clay, or to fasten strips of a metallic alloy around them or around entire plants, and let them sink to the bottom. In other cases seeds were sown.

RESULTS OF ECOLOGICAL INVESTIGATIONS

Salinity. The average salt content of the water at each of the 26 stations for a period of 48 consecutive months, beginning with August, 1925, is shown in Figure 6. These averages represent the salt content of surface samples and are presented in percentages of normal sea water, which ac-

cording to Clarke (21) contains 3.5 per cent salt. The salinity of Back Bay invariably was found lower than that of Currituck Sound. The salt content of the water was usually found to increase gradually from Back Bay through the connecting Knott's Island Channel to Currituck Sound and the Albemarle and Chesapeake Canal. The salinity was consistently higher near the southern end of the canal, averaging more than twice that of the center of Back Bay.

The salt content of the water varied greatly from month to month and from station to station. In August, 1925, for example, the salinity oft he water near the mouth of the Albemarle and Chesapeake Canal was 56.0

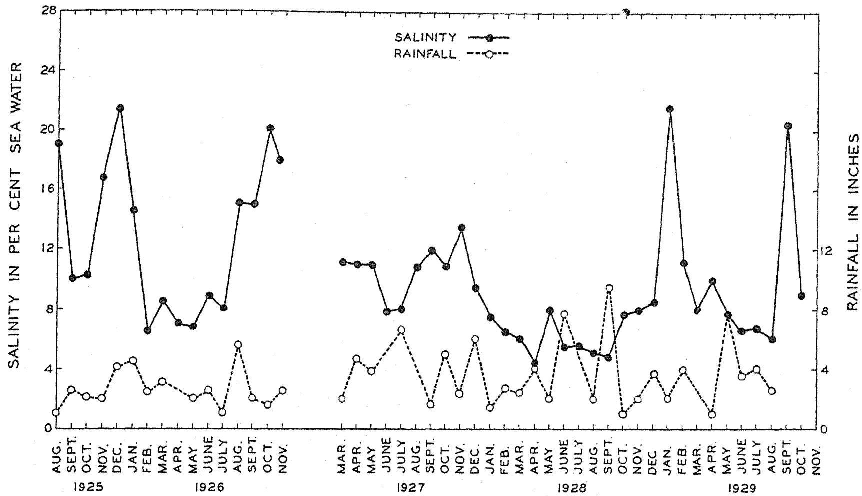

FIGURE 8. The influence of rainfall on the salt content of the waters of Back Bay and Currituck Sound. The salinity curve represents the average salt content in per cent sea water at 26 stations.

per cent sea water, but the following month it was only 14.6 per cent. For the same months the salt content of the center of Back Bay was 6.3 and 7.5 per cent, respectively. The lowest salt content determined in Currituck Sound was 3.2 per cent sea water, and the highest 66.0 per cent; the lowest in Back Bay 3.0 per cent, and the highest 20.0 per cent sea water. The salt content of North Bay did not vary greatly, being usually less than 5.0 per cent sea water. The average monthly variations in salinity for the total 26 stations were plotted and are shown in Figures 8 and 9 in connection with curves for rainfall and winds. The range of variations is shown to be from 4.5 per cent sea water in April, 1928, to 21.5 per cent in January, 1929. The variations are lowest for the seasons, at least the growing periods, of 1926 and 1928. Data on the salinity values for 1930

are incomplete, ending with results of analyses made on August 28, and are not presented. These do not, however, alter the averages for previous years.

The salinity of the bottom muds was found to vary little from that of the water above them. Soils of the marshes, however, varied considerably in salinity, a few containing a salt content equal to 30.0 per cent sea water and others no greater than 2.0 per cent. Soil samples containing the higher content were usually collected from more or less barren spots in the marshes. These saline spots in the marsh soils may have been caused by an overflow of salty water and its subsequent evaporation; there was no evidence obtained to show that they were caused by seepage of salt water from an underground source. Similar results were obtained from analyses of water seeping into holes dug in the barrier beach. Some samples of such water showed a salt content as high as sea water, or even higher, and others indicated a salt content as low as 4.0 per cent sea water. The wells of the region are all shallow and contain only surface water. Water from only two out of twelve wells tested showed more than a trace of chlorides, and these two were near the marshes. The salt content of one was 0.5 per cent sea water and the other 1.2 per cent. Water from a flowing artesian well in the marsh between Back Bay and Currituck Sound was found by Denny (25) to have a salt content of 60.0 per cent of sea water. This well, however, had been plugged before 1926.

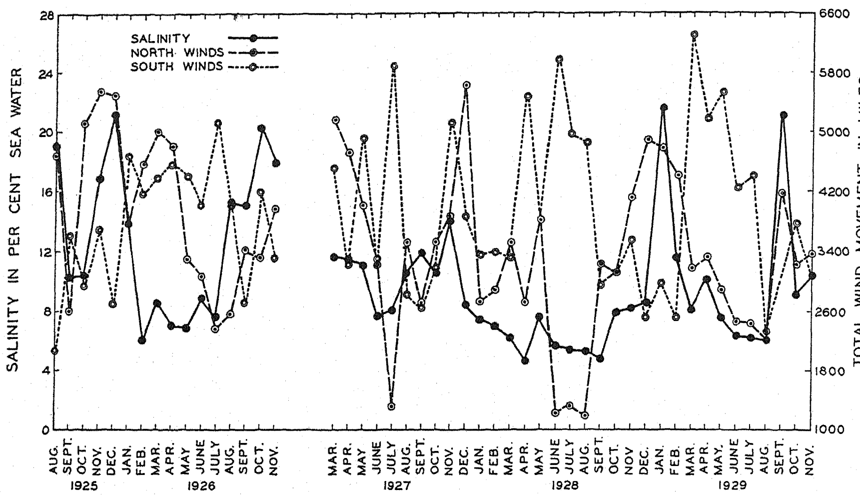

FIGURE 9. The influence of the direction, velocity, and duration of the winds on the salt content of the waters of Back Bay and Currituck Sound. The salinity curve represents the average salt content in per cent sea water at 26 stations.

Light. The average percentage transmission of total solar energy present at the surface is shown graphically in Figure 10 for water at various depths in North Bay, Back Bay, Knott's Island Channel (connecting Back Bay and Currituck Sound), and Currituck Sound. In each case the curve represents the average of 144 comparative readings taken at the particular depth during the year 1929. Forty-five such readings taken during 1930 did not appreciably alter the averages for the previous year and the data were not plotted.

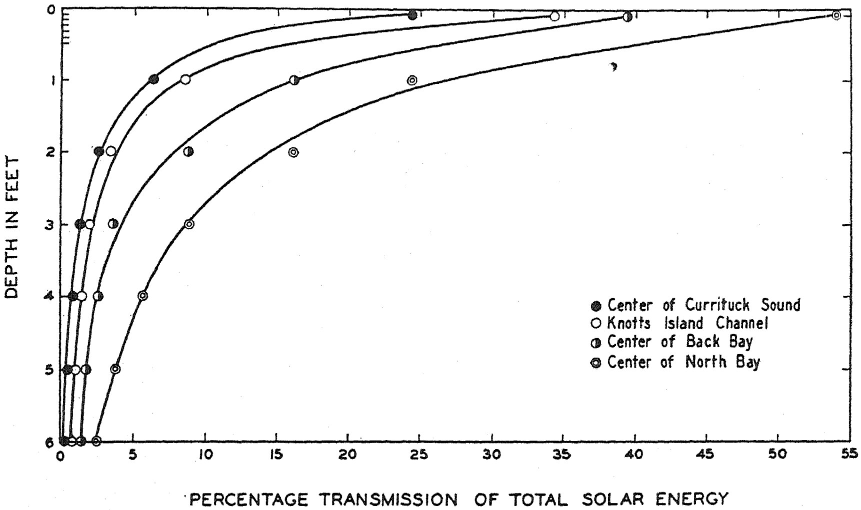

FIGURE 10. The percentage transmission of total solar energy in the waters of Back Bay and Currituck Sound. Points on the curves represent the average of 144 measurements made during the season of 1929.

During the season of 1929 the clearest water was found on September 9, when at six-foot depths the percentage transmission of the total solar energy present at the surface was 3.4 in North Bay, 2.3 in Back Bay, 1.0 in Knott's Island Channel, and 0.9 in Currituck Sound. The minimum percentage transmission was found on July 29, 1.8 at six feet in North Bay, 0.3 at six feet in Back Bay, 0.0 at five feet in Knott's Island Channel, and 0.0 at four feet in Currituck Sound. In 1930 the clearest water was found on July 23, when at six-foot depths the percentage transmission was 8.2 in North Bay, 5.0 in Back Bay, 3.6 in Knott's Island Channel, and 2.0 in Currituck Sound. The greatest turbidities, on the other hand, were recorded on August 28, when the percentage transmission was 1.4 in North Bay at a depth of six feet, 0.0 in Back Bay at five feet, 0.0 in Knott's Island Channel at five feet, and 0.0 in Currituck Sound at three feet.

TABLE I

DISSOLVED OXYGEN (PARTS PER MILLION) AT THREE FOOT DEPTHS IN THE WATERS OF BACK BAY, VIRGINIA, AND CURRITUCK SOUND, NORTH CAROLINA

Date 1930		Center of North Bay	Center of Back Bay	Knott's Island Channel	Center of Currituck Sound	Albemarle and Chesapeake Canal at Pungo Ferry
June	3	4.84	3.93	3.80	2.04	0.96
"	4	5.62	4.16	3.95	2.55	1.20
"	5	—	3.79	—	—	—
"	6	—	3.80	3.67	—	—
"	7	6.2[illegible]	3.20	3.43	1.79	0.67
"	10	6.27	4.12	3.70	2.52	1.00
"	12	6.98	4.18	4.10	3.00	1.70
"	13	6.82	5.60	4.43	3.60	1.90
"	14	—	5.08	4.06	—	—
"	17	6.00	2.34	3.09	—	—
"	18	6.50	1.79	1.28	—	—
"	19	6.63	3.30	3.00	2.24	1.60
"	20	—	—	—	1.60	1.31
"	23	6.73	3.20	2.88	1.55	1.12
"	24	—	3.00	2.65	—	—
"	27	6.74	2.33	2.10	1.28	0.85
"	28	—	2.40	2.24	1.60	1.40
"	30	5.60	3.20	—	—	—
Av.		6.24	3.49	3.22	2.16	1.25
Av. Temp., °C.		28.5	28.8	28.8	30.0	30.0
% Saturation*		80.0	45.0	41.0	28.0	16.0
July	1	—	3.30	2.40	—	—
"	2	4.80	3.81	3.00	3.48	2.70
"	3	—	3.90	3.13	3.43	2.65
"	7	5.70	4.48	4.16	—	—
"	8	—	4.80	—	—	—
"	11	6.30	4.70	4.10	4.48	2.70
"	12	—	4.72	4.10	4.43	2.65
"	14	6.73	4.18	3.60	3.13	2.14
"	15	—	3.93	2.88	3.01	2.10
"	17	6.30	3.30	2.40	—	—
"	18	6.30	3.20	2.28	1.97	1.01
"	19	6.62	3.60	2.10	1.60	0.96
"	21	—	3.20	—	—	—
"	23	4.80	3.00	2.40	1.28	1.20
"	24	4.90	3.00	—	—	—
"	25	—	4.48	—	—	—
"	28	6.89	5.70	5.28	5.60	4.40
"	31	6.85	6.00	4.70	4.48	3.00
Av.		6.01	4.05	3.18	3.35	2.43
Av. Temp., °C.		29.0	29.0	29.0	29.0	29.0
% Saturation*		77.0	52.0	41.0	43.0	33.0

* Per cent saturation after corrections were made for temperature and salinity.

TABLE I (*Continued*)

DISSOLVED OXYGEN (PARTS PER MILLION) AT THREE FOOT DEPTHS IN THE WATERS OF BACK BAY, VIRGINIA, AND CURRITUCK SOUND, NORTH CAROLINA

Date 1930		Center of North Bay	Center of Back Bay	Knott's Island Channel	Center of Currituck Sound	Albemarle and Chesapeake Canal at Pungo Ferry
August	1	6.32	5.62	4.39	4.30	2.70
"	2	—	4.80	4.10	4.00	2.40
"	5	5.98	4.78	—	—	—
"	6	—	4.70	3.72	3.43	—
"	7	5.10	4.61	3.41	3.00	2.10
"	11	4.70	4.43	3.09	1.79	2.00
"	12	—	3.93	3.00	—	—
"	13	—	4.10	—	—	—
"	14	4.78	4.43	3.70	3.13	2.70
"	18	6.00	4.90	3.93	3.41	2.70
"	20	5.81	4.49	3.60	3.09	2.55
"	21	—	4.54	3.80	3.43	2.30
"	22	—	4.43	—	—	—
"	23	—	4.48	—	—	—
"	26	4.84	4.45	4.20	4.16	—
"	27	4.70	4.60	4.10	4.00	2.70
"	28	5.60	4.48	4.12	4.00	2.50
"	30	6.00	4.54	4.43	4.40	2.33
Av.		5.44	4.57	3.82	3.55	2.45
Av. Temp., °C.		26.0	26.5	26.5	26.5	26.5
% Saturation*		66.0	56.0	47.0	43.0	31.0
September	2	5.80	4.10	4.10	4.00	2.70
"	3	—	4.78	4.41	4.00	2.65
"	4	—	5.60	4.00	3.60	2.34
"	5	6.00	5.28	—	—	—
"	8	—	5.15	5.00	3.72	3.00
"	9	6.00	5.60	5.10	3.93	3.09
"	10	—	—	—	4.01	—
"	11	—	—	5.00	4.12	—
"	12	—	4.98	4.54	4.00	3.01
"	13	5.60	4.10	4.00	3.72	—
"	16	5.00	4.09	3.78	3.43	2.70
"	18	5.81	4.48	4.40	3.60	2.00
"	19	—	5.00	4.78	4.00	2.65
"	22	6.63	4.49	4.43	3.60	2.33
"	23	—	4.01	3.79	3.60	—
Av.		5.83	4.74	4.41	3.81	2.65
Av. Temp., °C.		25.5	25.5	25.5	25.5	25.5
% Saturation*		70.0	58.0	54.0	50.0	38.8

* Per cent saturation after corrections were made for temperature and salinity.

It can be readily seen that the most turbid water is in Currituck Sound and the clearest in North Bay. The latter body of water is more remote from the Albemarle and Chesapeake Canal and at the time was the only water in the region containing appreciable quantities of aquatic plants.

Dissolved oxygen. The results of analyses for the determination of the content of dissolved oxygen are given in Table I. The amounts represent parts per million by weight, for samples obtained at three-foot depths in the various bodies of water listed. Averages for each of four months are given as parts per million and also as percentages of saturation after corrections were made for temperature and salt content of the water.

Analyses of water samples taken at six-foot depths in Back Bay usually showed a somewhat higher content of dissolved oxygen than analyses of those taken at a depth of three feet, but just the reverse was shown by analyses of samples taken at the same depths in Currituck Sound. In both bodies of water, however, only slight differences were shown by samples obtained from just under the surface and those taken at three-foot depths. For example, on June 27, a sample taken just below the surface of Back Bay contained a dissolved oxygen content of 2.34, one taken at three feet 2.33, and one taken at six feet (bottom) 4.16 parts per million. On the same date, a surface sample from Currituck Sound contained 1.31, one from a depth of three feet 1.28, and one from the bottom at six feet 1.0 parts per million dissolved oxygen.

The content of dissolved oxygen was invariably lowest in the Albemarle and Chesapeake Canal and highest in the water of North Bay where there was an appreciable quantity of aquatic plants throughout the season. Variations in the content of dissolved oxygen are seen to be greatest in the intermediate waters of Currituck Sound, Knott's Island Channel, and Back Bay. In these waters the variations sometimes exceeded 100 per cent in less than one week. For example, between July 23 and 28 there was a variation of 334 per cent.

Dissolved carbon dioxide. The results of occasional analyses made during the growing season always showed a content of dissolved carbon dioxide at the bottom in excess of 20 parts per million by weight except in the water of North Bay where the content ranged from 4 to 10 parts per million near the bottom to 2 parts per million or less at the surface. In the main parts of Back Bay and Currituck Sound the content of dissolved carbon dioxide in parts per million varied from 20 to 30 at the bottom to 4 to 6 near the surface. In the shallow waters over soft, muddy bottoms, containing a great amount of decomposing organic matter and barren of seed plants, the content frequently was found to be as high as 42 parts per million at the bottom and 12 at the surface.

Hydrogen ion concentration. The hydrogen ion concentration, as shown by the quinhydrone electrode method, varied only slightly during a sea-

son, or from one season to another. The water in Back Bay and Currituck Sound was always found to be practically neutral, ranging from pH 6.2 to 6.8. In North Bay, however, the water near the surface, because of the diminution of carbon dioxide by vegetational activity, usually reacted alkaline to phenolphthalein.

Temperature. The average water temperatures for the summer 1930 are given in Table I. These averages represent temperatures at three-foot depths and were determined at the time samples were obtained for analyses of dissolved oxygen content. No winter temperatures were recorded during the investigation, but the larger bodies of water are seldom known to freeze over during an entire winter.

While the surface water of shallow ponds in the marsh, especially of those not containing seed plants, frequently exceed 36° C., the highest temperature recorded for the open water was that for the center of Currituck Sound on July 28, 1930, at 2:00 p.m. With the thermometer bulb just under the surface a temperature of 35.5° C. was recorded, at six inches 36.3° C., at one foot 35.7° C., at three feet 33.8° C., and at six feet (the bottom) 33.0° C. There was a considerable breeze at the time which probably accounts for the surface being somewhat cooler than the water a few inches below it. Usually in summer the temperature of the water diminished from the surface towards the bottom approximately 1° C. for each foot in depth. Often only slight differences were found, however, between surface and bottom temperatures in shallow waters containing submerged plants in appreciable quantities. For example, at midday on August 22, the temperature just below the surface of a pond having a luxuriant plant growth was 26.0° C. and on the bottom at a depth of three feet it was 25.0° C.

Atmospheric temperatures for several days during midsummer were frequently recorded as high as 41.0° C. in the shade. This is about 2.0° C. higher than the absolute maximum temperature given by the Norfolk station of the U. S. Weather Bureau.

Rainfall and winds. The monthly amounts of rainfall in inches from August, 1925, to October, 1929, inclusive, are presented graphically in Figure 8 in connection with a curve representing the average salinity of Back Bay and Currituck Sound for the same months. The broken line represents rainfall in inches and the solid line the average salinity in per cent normal sea water (3.5 per cent total salts). It can be readily seen that the amount of rainfall had little effect upon the salinity of the water. For example, in December, 1925, the average salt content was 21.15 per cent sea water, and the rainfall was 4.20 inches during the month. This was more than the normal amount, 3.49 inches, for December in the region. Furthermore, this amount of rainfall was distributed over ten days of the month, which is considered a normal distribution. Then, in April, 1928

there was practically the same amount of precipitation 4.28 inches, as in December, 1925, and distributed over ten days of the month. Yet, the average salt content of the water during April, 1928, was only 4.65 per cent sea water. The coefficient of correlation between amount of rainfall and the salinity of the water in Back Bay and Currituck Sound is −0.2495, with a probable error of ±0.1331, which is considered insignificant.

The relationship between winds and the average monthly salinities of Back Bay and Currituck Sound is brought out graphically in Figure 9. Wind movement and salinity averages for 48 months are given. In the region there is little wind movement directly from the east or west and this has been disregarded in the calculations. North, northeast, and northwest winds have been considered as north winds, and south, southeast, and southwest winds as south winds in the graph. The direction, velocity, and duration of the winds are shown to have a marked influence on the salinity of the water. North winds, even of average velocity, sustained for relatively short periods of time cause a southward flow of salt water from Norfolk Harbor through the Albemarle and Chesapeake Canal and thus increase the salt content of Currituck Sound and Back Bay in proportion to the flow. On the contrary, south winds cause an opposite flow through the canal and, consequently, dilute the salt content of these waters by forcing in fresh water from the south. The influence of winds on the variations in salt content of the water is observed to be greater than that of rainfall. For example, this is brought out strikingly by using the same months for comparison as were used to show the influence of rainfall. In December, 1925, the prevailing winds were somewhat west of north, the wind with the maximum velocity was from the northeast at 56 miles an hour, the total wind movement for the month from all directions was 10,441 miles, of which 5,399 were from the north, and the average salt content of the water of Back Bay and Currituck Sound was 21.15 per cent sea water. In April, 1928, however, the prevailing winds were from the south, the wind with the maximum velocity was from the southwest at 40 miles an hour, the total wind movement was 10,210 miles, of which 5,430 were from the south, and the average salt content of the water of Back Bay and Currituck Sound was 4.65 per cent sea water. Direct correlation can be observed in Figure 9 between the curves representing wind movement and the curve denoting the average salt content of the water. The correlation coefficient between north winds and salinity is +0.3729, with a probable error of ±0.0901, which is a very significant correlation, as the odds are more than 100 to 1. The correlation coefficient between south winds and salinity is −0.3420, with a probable error of ±0.0883, which again is quite significant.

Experimental plantings. Despite the fact that many thousand speci-

mens of *Potamogeton pectinatus*, *Potamogeton perfoliatus*, and *Vallisneria spiralis*, in the form of entire plants, tubers, rhizomes, and seeds, were obtained from fresh and brackish waters over a wide range in the eastern half of the United States and transplanted in the region, no plants were observed to grow in the open water or in ponds connected to Back Bay by open ditches. When coves and ponds were inclosed by bulkheads and dikes and thus protected from the open water of Back Bay and Currituck Sound, however, luxuriant growths of all species of submerged seed plants inhabiting the region were obtained, except in the case of a few small, shallow ponds. All plants seemed to grow in the larger inclosed bodies of water regardless of the form of the plants or the method of transplanting them. Figure 7 illustrates a luxuriant growth of submerged seed plants in water four feet deep, thriving on the natural bottom of a cove approximately 100 acres in area. In that part of the area appearing in the photograph a natural growth of plants returned soon after the completion of the bulkhead on the right. This bulkhead is not water-tight, but prevents entrance into the cove of the sediment and suspended matter of the extremely turbid water of Back Bay. The water in this cove has been clear and transparent since the erection of the bulkhead, but analyses show that there is no difference between its salt content and that of the water in Back Bay. The level of the water in the cove remains the same as that of the water on the other side of the bulkhead.

In an artificial pond, 100 acres in area, plant growth was only slowly established. This pond had been dredged six feet in depth and into a blue-clay, marsh subsoil. It was found after unsuccessful efforts at propagation that such a bottom would not support a growth of submerged seed plants, but whether this was because it was too hard for the plant roots to penetrate or through the lack of essential plant nutrients was not determined. Top soil from the surrounding marsh was then blown into the pond by the use of high explosives. In addition, a ditch was dug in order to connect the pond to Back Bay and permit the entrance of sediment. After a year the bottom of the pond was found to be covered with several inches of soft mud. The ditch was then closed by bulkheads. The water in the pond remained as turbid as that of Back Bay for a few months until growths of *Chara* spp. and *Nitella* spp. almost completely blanketed the bottom. Yet, the growth of these plants did not interfere with the successful propagation of submerged seed plants after the water cleared.

The inclosing of small, shallow ponds was found to prevent the free circulation of water to such an extent that the temperature of the water in midsummer frequently rose higher than 38.0° C. At this temperature photosynthetic activity of the plants ceased, the content of dissolved oxygen dropped to less than two parts per million, and death of the plants

soon followed. In these small ponds only a temporary stand of *Naias flexilis* was secured after the water began to cool to less than 30.0° C. in late summer and early autumn.

PHYSIOLOGICAL INVESTIGATIONS

MATERIALS AND METHODS

Salinity. To determine the effect of different concentrations of sea water on the growth of *Potamogeton pectinatus* L., *Potamogeton perfoliatus* L., *Vallisneria spiralis* L., *Naias flexilis* (Willd.) Rostk. & Schmidt, and *Ceratophyllum demersum* L., experiments were carried on in the greenhouse from January 20 to March 20. Ten terminal, unbranched cuttings, 15 cm. in length, of each species, except *Vallisneria spiralis*, were weighed and placed in tap water and 10 cm. of soil contained in stoneware vessels of approximately 45 liters capacity. In experiments with *Vallisneria spiralis*, ten winter buds, each about the same in size and weight, were employed. The plant material used in these experiments was taken from plant cultures grown in the greenhouse. All plants, except *Potamogeton pectinatus* which was obtained from Minnesota, were originally collected from Back Bay, Virginia. After a week, when the plants had become attached in the soil and the initiation of growth was observed, the tap water was siphoned from the cultures and replaced by various concentrations of Long Island Sound water diluted with tap water. Long Island Sound water, which was found to be 80 per cent sea water, was diluted with tap water to make the following concentrations of normal sea water (3.5 per cent total salts) for each species in separate cultures: 0, 2, 4, 8, 12, 16, 20, 24, 28, 32, and 36 per cent sea water. The solution in each culture vessel was renewed every second day. The temperature of the solutions varied during the experiment from 20° to 22° C. Concentrations of dissolved carbon dioxide and oxygen of approximately 60 and 8 parts per million by weight respectively, were maintained fairly constant in each culture throughout the experiments. This was accomplished by bubbling the gases through Berkfeld filter cores into the solutions and checking the concentrations by the methods of the American Public Health Association (2). Artificial illumination was supplied for four hours each day after sundown from 1500 watt electric bulbs and reflectors suspended about three feet above the cultures. A photoelectric cell and galvanometer indicated that approximately 500 foot candles of artificial light were thus supplied at the surface of each culture vessel. The temperature of the solutions was maintained approximately at 20° C. After eight weeks the experiments were discontinued, the plants removed, and carefully washed and brought to a constant dry weight in an oven at 60° C. The increase in dry weight in each case was then calculated by comparing with the dry weight of comparable samples taken at the beginning of the experiments.

Light. To determine the effect of shading on the growth of *Potamogeton pectinatus* L., experiments were conducted during the year 1929 in the greenhouse. Eleven tanks, constructed of heavy cypress lumber, were used as culture vessels. These tanks were four feet in length and three feet in width. Four of them were six feet, four were five feet, and three were four feet in depth; these had approximate capacities of 538, 448, and 369 gallons, respectively. The deeper tanks were imbedded in the floor of the greenhouse to depths sufficient to bring their surfaces even with those of the tanks four feet in depth. Each tank was fitted with an outlet pipe near the bottom and an inlet pipe at the top. These pipes were connected by valves to pipe lines leading to and from an electric pump which circulated water in the tanks or from one tank to another, as desired. Overflow pipes from the surface of the tanks led outside the greenhouse to a tank, similarly connected to the circulation system, for the purpose of aerating the water. The main pipes of the circulation system passed through a large cylinder fitted for heating the water with steam. The circulation system, therefore, provided not only for the aeration of the water but also for the regulation of its temperature. In addition, the circulation of the water was found to interfere with the growth of algae in the cultures.

On March 1 a six-inch layer of good garden soil was placed in each tank which was then filled with tap water. Eleven sets of 48 *Potamogeton pectinatus* tubers, weighing approximately 45 grams per set, were planted in rows in the soil contained in the eleven tanks. The tanks six feet in depth were then shaded as follows: one tank was left open for a control experiment, one was covered with a layer of mosquito netting, one with a layer of cheesecloth, and one with a layer of muslin. Tanks five feet in depth were treated similarly except the mosquito netting was omitted and instead one tank was covered with two layers of muslin. The three tanks four feet in depth were treated as follows: one tank was left unshaded to serve as a control experiment, one was covered with one layer of muslin, and the remaining one was shaded with two layers of muslin. The water in the tanks was circulated daily and renewed once a week. The temperature of the water was maintained approximately at 23° C. The transmission of total solar energy through the different coverings and the water to the bottom of each tank was measured from time to time with a Shirley thermoelectric radiometer. The experiments were concluded on September 28, and the plants harvested, washed, and brought to constant dry weight in an oven at 60° C.

Dissolved gases, substrata, and nutrient solutions. In order to determine the effect of various concentrations of dissolved carbon dioxide and oxygen on the growth of submerged seed plants and the dependence of their growth on the substratum, three series of experiments were performed in duplicate. Cylindrical stoneware vessels of about 45 liters capacity were

used as culture vessels. Twenty-seven separate cultures were employed in a series, each of which was divided into nine sets of three cultures. Each set was treated as follows: one vessel was supplied with a layer of good garden soil 10 cm. thick, a second with a layer of pure, acid washed, quartz sand of the same thickness, and no substratum was added to the third vessel. All vessels were then filled with tap water. The plants selected for experimental material in each culture were ten terminal unbranched cuttings, each 15 cm. in length. These cuttings were without roots and were selected from stock plants which had been growing in the greenhouse aquaria for several years. Cuttings were taken from plants approximately uniform in size. In each set of three cultures, ten cuttings were placed with their basal ends up to the first node in soil, ten similarly in quartz sand, and ten were anchored with paraffined cotton threads and metallic weights at a level in the vessel containing no substratum equal to the level of the plants in the vessels containing soil and sand. In each series of experiments, therefore, there were nine sets of ten plants each treated in this manner.

Free carbon dioxide and oxygen were supplied from cylinders containing the gases. The carbon dioxide used was the product of fermentation in a process connected with the refining of cane sugar and the manufacture of ethyl alcohol. The ordinary commercial carbon dioxide, manufactured by the combustion of coke, was found toxic, probably because of gases contained in it as impurities, and could not be used with cultures of aquatic plants. The carbon dioxide and oxygen were bubbled into the culture solutions through Berkfeld filter cores extended by glass tubing to the bottoms of the culture vessels. To one set of three cultures in each series carbon dioxide was added occasionally during the daytime to maintain a concentration of the dissolved gas equivalent to 72 parts per million by weight. Dissolved contents of 24 parts per million were maintained in a second, and 8 parts per million in a third set. The concentrations were regulated and checked every three hours by the titration methods of the American Public Health Association (2). No oxygen was added to these three sets. Another set of cultures, however, received carbon dioxide in the daytime in sufficient quantities to maintain a concentration of the dissolved gas in each culture equivalent to 72 parts per million and oxygen during the night equivalent to a dissolved content of 28 parts per million by weight. Dissolved contents of 24 parts per million carbon dioxide and 14 parts per million oxygen were similarly maintained in a second set, and 8 and 14 parts per million of carbon dioxide and oxygen, respectively, in a third set. The concentration of carbon dioxide was regulated only during the daytime, as before described, and that of the oxygen only during the night, every three hours, by testing the concentration according to the Winkler method. Determinations of both carbon dioxide and oxygen concentrations, however, were made every three hours during the day and night.

In a like manner, one set of cultures received oxygen without carbon dioxide sufficient to maintain during the night a dissolved content in the cultures equivalent to 28 parts per million, and another set received 14. These concentrations of oxygen were kept fairly constant during the night but not during the day. A final set of three cultures in each series served as control cultures and received no additions of carbon dioxide and oxygen. These control cultures, however, were tested regularly every three hours during the day and night for their contents of dissolved carbon dioxide and oxygen.

In the experiment there were two series of cultures, one containing tap water and the other Sachs' nutrient solution, diluted in the proportion of one volume of the solution, as ordinarily made up, to 30 volumes of tap water. The culture solutions were renewed every second day, and their temperatures kept between 23° and 25° C.

The first two series of experiments were carried on from March 31 to April 28, 1931, with cuttings of *Naias flexilis* (Willd.) Rostk. & Schmidt. Ten cuttings were selected for each culture, the excess water adhering to plants removed, and the total fresh weight of the plants determined. The cuttings were then placed in the culture vessels according to the methods previously described before they were allowed to dry in the air to the point of injury. At the time of selecting the cuttings for the experimental cultures, comparable samples of ten cuttings were chosen for the determination of initial dry weights to serve as a basis for the calculation of increase in dry weight of the cuttings used in the experiments. After the excess water adhering to the plants had been removed, the total fresh weight of the cuttings was determined and the samples brought to a constant dry weight in the oven at 60° C. After four weeks the plants in the experimental cultures were carefully removed in order to prevent breaking off the roots, washed, the increase in growth of stems, new branches, and roots measured, and the plants dried to a constant weight in the oven at 60° C. The second and third series of duplicate experiments were carried out in a similar manner from June 12 to July 10, 1931, with cuttings of *Potamogeton perfoliatus* L. and *Potamogeton foliosus* Raf., respectively. In all experiments the hydrogen ion concentration of the different solutions was determined by the glass electrode and colorimetric methods before and after renewing the solutions.

For the purpose of determining the effect on the growth of submerged plants of nutrient solutions containing nitrogen in different forms, a second experiment with *Potamogeton perfoliatus* L. and *Naias flexilis* (Willd.) Rostk. & Schmidt cuttings was carried on from December 7, 1931, to January 4, 1932. The cultures were arranged, as previously described, in sets of three, one containing a soil, one a sand, and one no substratum. The same number of cuttings were employed and treated in a manner sim-

ilar to that already described. Sachs' nutrient solution, diluted with tap water in the proportion of one volume of the normal solution to 30 volumes of tap water, was used as a nutrient solution containing nitrogen in the form of nitrate. Before dilution this solution was made up of 1.0 gram potassium nitrate, 0.5 gram each of calcium sulphate, magnesium sulphate, and calcium phosphate, and two drops of a ten per cent solution of ferric chloride to one liter of water. The hydrogen ion concentration of such a solution, as determined by the glass electrode method, is approximately pH 6.5. A second nutrient solution was made up in a similar manner, except 0.654 gram of ammonium sulphate and 0.853 gram of potassium sulphate were substituted for the 1.0 gram of potassium nitrate in Sachs' solution. Thus, the second solution contained the same amounts of nitrogen and potassium as Sachs' solution, but the nitrogen was in the form of ammonium instead of nitrate. In the second solution the amount of sulphate ions and the ionic concentration were only very slightly increased. There was little difference between the hydrogen ion concentrations of the two solutions. The second solution was diluted with tap water in the same proportions as the first. A third solution was made up to contain nitrogen in the form of both nitrate and ammonium by combining equal volumes of the diluted first and second solutions. Each of the three solutions was then added to two sets of the plant cultures, in one set of which a concentration of 72 parts per million of dissolved carbon dioxide was maintained during daylight throughout the experiment. The other half of the culture sets contained only the nutrient solutions without added carbon dioxide. All solutions were renewed in the cultures every second day. Artificial illumination was supplied daily for four hours directly after sundown in the same quantity and manner as described previously for the experiments on the effect of salinity. The temperature of all solutions was maintained at 23° to 25° C.

An additional experiment was conducted from April 18 to May 16, 1931, for the purpose of determining the rooting response of *Naias flexilis* cuttings under various conditions. Nine terminal cuttings, without roots, 15 cm. in length were used in each of four cultures contained in glass museum jars of about six liters capacity. Two of the jars were filled with the diluted Sachs' nutrient solution and two with only tap water. No culture contained a substratum. One jar containing Sachs' nutrient solution and another only tap water were shaded so as to exclude all the light except that falling directly on the narrow surface at the top of the vessels. The two remaining jars were exposed on all sides to the full light of the greenhouse. A small quantity of carbon dioxide was bubbled daily into the solutions, which were renewed every morning. The temperature of the solutions was kept fairly constant at 21° C. Three plants in each culture were anchored near the bottom of the vessels by attaching to their basal nodes

a short paraffined cord tied to a small piece of glass rod; three had their basal nodes attached by means of paraffined cords directly to smooth glass rods suspended near the bottom of the vessels; and the remaining three plants in each culture had their basal nodes attached to glass rods in a similar manner, except the glass rods previously had been dipped first into celloidin and then quickly into clean quartz sand and allowed to dry before the plants were attached.

RESULTS OF PHYSIOLOGICAL INVESTIGATIONS

Salinity. The effect of different concentrations of sea water on the growth of *Potamogeton pectinatus* L., *Potamogeton perfoliatus* L., *Vallisneria spiralis* L., *Naias flexilis* (Willd.) Rostk. & Schmidt, and *Ceratophyllum demersum* L., under favorable conditions of soil, carbon dioxide supply, light, and temperature, is shown in Table II. The results are presented in

TABLE II

EFFECT OF DIFFERENT CONCENTRATIONS OF SEA WATER ON THE GROWTH OF POTAMOGETON PECTINATUS, POTAMOGETON PERFOLIATUS, VALLISNERIA SPIRALIS, NAIAS FLEXILIS, AND CERATOPHYLLUM DEMERSUM. JANUARY 20 TO MARCH 20, 1932

Percentage sea water	Percentage increase in dry weight				
	Potamogeton pectinatus	*Potamogeton perfoliatus*	*Vallisneria spiralis*	*Naias flexilis*	*Ceratophyllum demersum*
0	1230	3100	361	1300	4800
2	1455	4432	322	1415	2778
4	1677	5333	290	1530	2365
8	2122	8440	230	1030	1954
12	2466	9933	166	600	1400
16	2689	4860	84	248	995
20	2967	4567	38	16	440
24	2788	4166	0	4	12
28	1744	3500	0	0	0
32	380	412	0	0	0
36	96	290	0	0	0

percentage increase in dry weight, using as a basis of calculation the dry weights of comparable samples taken at the beginning of the experiment. Increasing concentrations up to 28 per cent sea water stimulated the growth of *Potamogeton pectinatus* and *Potamogeton perfoliatus*, with the optimum concentrations at 20 and 12 per cent sea water, respectively. Growth of *Naias flexilis* was only slightly stimulated by concentrations of 2 and 4 per cent sea water, but that of *Vallisneria spiralis* and *Ceratophyllum demersum* was retarded decidedly by any concentration employed.

While the growth of *Potamogeton pectinatus* is checked considerably by concentrations of 32 and 36 per cent sea water, the plants grew well, developed abundant roots, appeared a normal green in color, and produced a normal crop of seeds. The undiluted water from Long Island Sound,

which contains a salt content equivalent to about 80 per cent sea water, was found not to plasmolyze the cells of this plant in 24 hours, in spite of the fact that the plant had been growing in fresh water.

The *Potamogeton perfoliatus* plants used in the experiment were small leaf forms, the leaves not exceeding 2 cm. in width and 3 cm. in length. The cuttings were taken from greenhouse stock, collected originally in Back Bay, Virginia. This plant grew well, produced abundant roots and seeds, and appeared perfectly normal in concentrations up to 32 per cent sea water, but in 32 and 36 per cent the plants grew spindling and suffered a marked reduction in the size of the leaves. In propagating this plant from cuttings it displays a characteristic not observed in any of the other submerged plants used in the experiment. The original cutting soon ceases to grow, and usually dies above the basal node. From this basal node, however, runners and new shoots originate within a week. In connection with the growth of this plant in sea water, it was found that concentrations less than 50 per cent would not plasmolyze its cells in 24 hours.

Vallisneria spiralis plants did not grow so well in any dilution of sea water used as in tap water. The winter buds of this plant, however, are rather dormant and the plant makes little growth in the greenhouse during the winter, although the buds germinated in every culture in the experiment. In concentrations of 16 and 20 per cent sea water the young plants appeared etiolated and the leaves were extremely narrow. The young plants died soon after germination in higher concentrations of sea water, yet it was found that concentrations less than 45 per cent sea water would not plasmolyze cells of the leaf.

The growth of *Naias flexilis* was insignificant in concentrations above 16 per cent sea water. In 20 per cent the plants were etiolated and quite spindling, and most of the original leaves turned yellow and dropped off. Plants in higher concentrations than 24 per cent sea water soon died and disintegrated. It required concentrations higher than 40 per cent sea water, however, to plasmolyze the cells of the plant in 24 hours.

While a decrease in the growth of *Ceratophyllum demersum* is indicated roughly in proportion to the increase in concentration of sea water, the plants appeared to develop normally in concentrations under 20 per cent. In 20 per cent sea water, however, there was a decided reduction in the size and number of leaves, which exhibited a tendency to curl, and the stems were very spindling. Twenty-four per cent sea water marked the limit of endurance for the plants. In higher concentrations the plants died and completely disintegrated within a week. It required, however, concentrations of sea water stronger than 35 per cent to plasmolyze the plant cells in 24 hours.

Light. The effect of depth of water and degree of shading on the growth of *Potamogeton pectinatus* L. is shown in Table III. The experimental re-

sults are expressed in absolute quantities of oven-dried material. It is seen that increasing the depth of water or increasing the degree of shading caused a decrease in the dry weight of the plants. The percentage transmission of total solar energy through the various coverings and the water to the bottom of the tanks[3] is illustrated graphically in Figure 11. Open tanks four feet in depth (Table III) received on the average at the bottom about 12.0 per cent of the total solar energy present outside the greenhouse, those five feet in depth 10.5 per cent, and those six feet in depth 9.5 per cent. The limit of growth is seen to lie between 2.5 and 3.5 per cent total solar energy. Growth declines rapidly, however, below 4.0 per cent total solar energy.

TABLE III

EFFECT OF DEPTH OF WATER AND AMOUNT OF SHADING ON THE GROWTH OF POTAMOGETON PECTINATUS L. MARCH 1 TO SEPTEMBER 28, 1929

Depth of tank, in feet	Tank covering	Dry weight of plants, in grams
4	2 layers muslin	0.00
4	1 layer muslin	12.50
4	Open tank (check)	351.00
5	2 layers muslin	0.00
5	1 layer muslin	0.30
5	1 layer cheesecloth	147.80
5	Open tank (check)	299.40
6	1 layer muslin	0.06
6	1 layer cheesecloth	80.80
6	1 layer mosquito netting	178.60
6	Open tank (check)	193.20

All tubers planted in the tanks germinated and the plants in covered tanks six feet in depth reached the surface within six weeks, about two weeks before those in the open control tank. The plants growing in the tanks shaded with mosquito netting and cheesecloth appeared perfectly normal in every way. The growth was uniform and thrifty. The internodes were short and the stem and leaves flexible, and those at the surface were typical of the forms developed at the surface of open tanks or in nature. The stems and leaves of these plants, however, were somewhat fewer and coarser than the dense growths of stems and hair-like leaves of the plants growing in the open tanks, and the foliage was a lighter green in color than that of the control plants. On the other hand, plants growing in the tanks covered with one or two layers of muslin were much etiolated. Their internodes were more than twice as long as those of the plants growing in the open tanks. Their stems were rigid and unbranched. Their leaves were few, coarse, and rigid. The tip of the stems and the terminal leaves exhibited a tendency to protrude and wilt above the surface of the water, in

sharp contrast to the flexible, floating stems and leaves of the control plants. No floating leaves were produced by these etiolated plants, all being typical underwater forms. With very few exceptions all the plants which germinated in the tanks shaded with muslin began to decay near the base

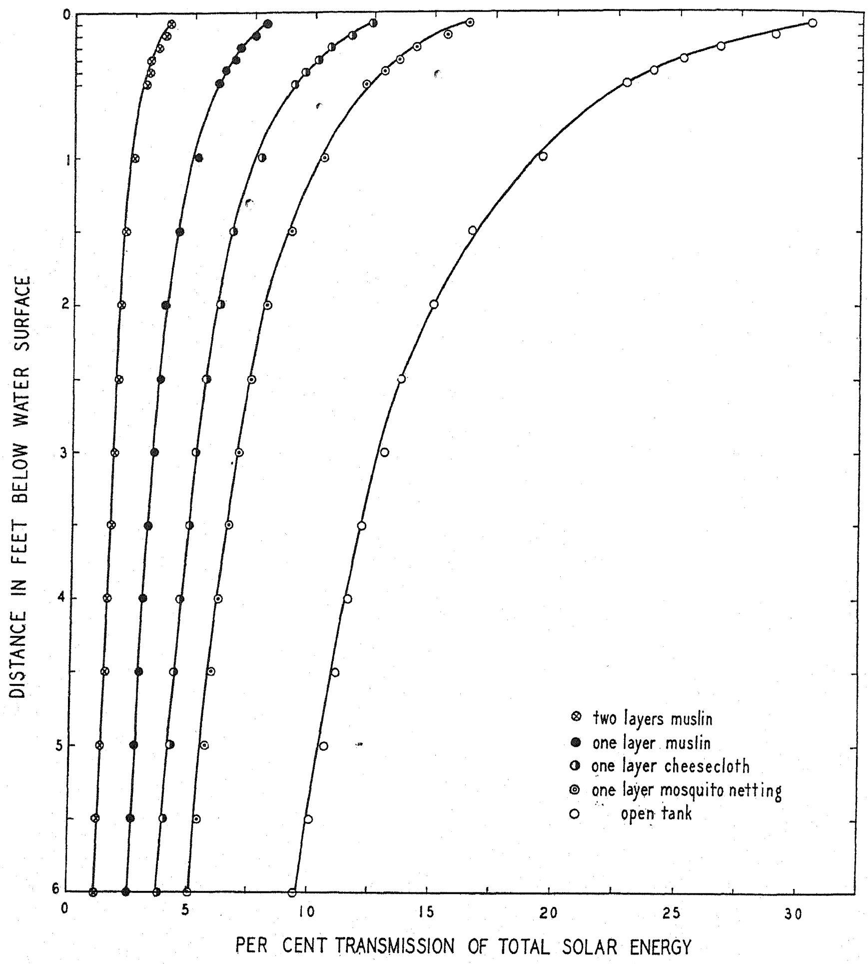

FIGURE 11. The percentage transmission of total solar energy in the water of greenhouse tanks shaded with various coverings. The points on the curves represent the average of six measurements made at midday on April 23, 1930.

of the stems soon after the plant tips reached the surface of the water and completely disintegrated within a few weeks. Those surviving at the conclusion of the experiment were hardly more than etiolated fragments.

Dissolved gases, substrata, and nutrient solutions. The effect of different

concentrations of dissolved carbon dioxide and oxygen on the growth of *Naias flexilis* (Willd.) Rostk. & Schmidt in dilute Sachs' solution and in tap water, with substrata of soil and sand, and without a substratum, is shown in Table IV. In most cases growth is seen to be considerably better in the Sachs' nutrient solution series than in the tap water series; better

TABLE IV

EFFECT OF DIFFERENT CONCENTRATIONS OF CARBON DIOXIDE AND OXYGEN ON THE GROWTH OF NAIAS FLEXILIS IN VARIOUS CULTURE MEDIA. MARCH 31 TO APRIL 28, 1931

Content dissolved gases* (p.p.m.)		Addition to liquid	Sachs' nutrient solution diluted 1 to 30			Tap water		
CO_2	O_2		Total gain in length, cm	Total length of roots produced, cm.	Increase in dry weight,** %	Total gain in length, cm.	Total length of roots produced, cm.	Increase in dry weight,** %
72		Soil	935	153	252	79	83	73
72		Sand	180	115	91	96	273	73
72		None	229	115	92	14	29	4
24		Soil	567	196	199	131	137	87
24		Sand	386	201	116	122	147	85
24		None	208	166	80	20	0	7
8		Soil	363	185	142	367	75	97
8		Sand	339	202	98	132	47	62
8		None	191	99	52	71	13	11
72	28	Soil	1767	440	267	216	132	92
72	28	Sand	446	156	129	97	97	44
72	28	None	217	97	82	47	46	25
24	14	Soil	546	204	201	306	65	91
24	14	Sand	562	276	184	181	53	69
24	14	None	260	107	125	65	8	31
8	14	Soil	377	175	155	447	208	129
8	14	Sand	303	186	76	79	86	78
8	14	None	184	77	57	65	27	32
	28	Soil	208	106	46	108	161	39
	28	Sand	114	56	30	18	54	17
	28	None	202	110	42	20	0	5
	14	Soil	371	159	147	307	98	95
	14	Sand	233	126	66	55	25	31
	14	None	180	45	54	15	0	8
Ck.	Ck.	Soil	383	137	149	248	118	109
Ck.	Ck.	Sand	194	147	71	64	89	22
Ck.	Ck.	None	165	101	52	9	20	3

* Amounts of CO_2 and O_2 recorded in this column were kept as constant as possible throughout the day and night, respectively. In cultures for which no values are listed gases were not added, but the amounts in these varied with the medium, photosynthesis, respiration, etc. See text for explanation.

** Initial dry weight was calculated from comparable samples at the beginning of the experiment.

in a soil substratum than in sand, or in cultures without a substratum; and better in the cultures to which appreciable quantities of carbon dioxide had been added. In the Sachs' nutrient solution series, the best growth, considering increase in length of plants and roots produced, occurred in soil cultures in which concentrations of 72 and 28 parts per million dissolved carbon dioxide and oxygen, respectively, were maintained. The growth in this culture, however, considered from the standpoint of dry weight produced, was little better than that in the soil culture in which a concentration of 72 parts per million dissolved carbon dioxide, without oxygen, was maintained, the difference being only about five per cent. Generally, the addition of oxygen is shown to have only a very slight influence on the growth of the plants, but when added without carbon dioxide it tended to retard growth. In cultures to which only carbon dioxide was added, the plants in light produced by their own photosynthesis more than a sufficient quantity of oxygen for their requirements, and further additions of oxygen to the solution retarded growth. In the tap water series, growth in only one culture surpassed that in the control culture in soil and tap water, the plants grew better in a soil culture in which concentrations of 8 and 14 parts per million of dissolved carbon dioxide and oxygen, respectively, were maintained. Growth in the tap water series apparently was retarded by higher concentrations of these dissolved gases. The plants which were merely anchored in tap water developed very few roots and made practically no growth, although the plants remained normal in appearance throughout the experiment. Plants grown in a sand substratum usually developed about as many roots as those grown in soil, and root hair development was more abundant in the former. The few roots developed by plants which were merely anchored in tap water and Sachs' solution had no root hairs. Root development of *Naias flexilis* cuttings grown in tap water (A), in tap water with a quartz sand substratum (B), and in tap water with a soil substratum (C) is shown in Figure 12.

Where no values have been recorded for dissolved carbon dioxide and oxygen in the first and second columns of Table IV, there was no gas added, but carbon dioxide and oxygen were always contained in these cultures. In the cultures to which no carbon dioxide was added the concentration of dissolved carbon dioxide at the beginning of the experiment was about 18 parts per million in the solutions with a soil substratum, and 5 parts per million in the solutions with sand and without a substratum. With increased growth of the plants the quantity of dissolved carbon dioxide diminished in the solutions over soil to 3 parts per million during the day and 5 parts per million during the night. Near the end of the experiment usually no dissolved carbon dioxide could be detected in the solutions with a sand substratum or in the solutions without a substratum during the day, and only a trace was found at night. Dissolved oxygen,

on the other hand, was always present in all the cultures in quantities no less than the amount of the gas contained in tap water. At the beginning of the experiment the solutions contained about 7 parts per million dissolved oxygen. Near the end of the experiment, just before a renewal of the solutions, the quantity of the gas present in the solutions was proportional to the growth of the plants. In cultures containing the greatest growth of plants the concentration of dissolved oxygen was found frequently to be as high as 17 parts per million during the day. This quantity usually decreased slowly during the night to about 14 parts per million

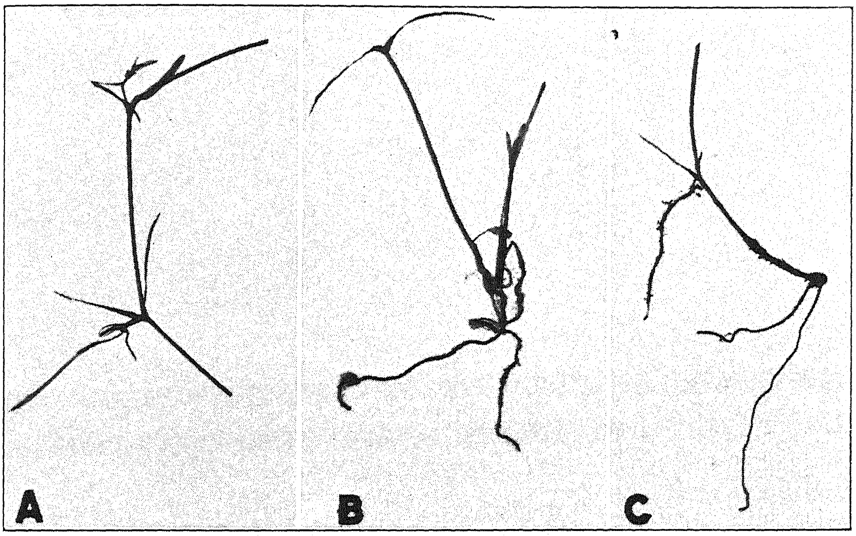

FIGURE 12. Rooting response of *Naias flexilis* cuttings: (A) Grown for four weeks anchored in tap water; (B) Rooted in tap water and quartz sand; (C) Rooted in tap water and garden soil.

at five o'clock in the morning. In those cultures which supported small plant growth the concentration of the gas was considerably less and the variations in the quantity present during the day and night were comparatively slight. In some of the cultures receiving 24 and 8 parts per million carbon dioxide and 14 parts per million oxygen, it was often found necessary to change the solutions daily in order to maintain the concentration of dissolved oxygen at 14 parts per million, which was a lower concentration than that being supplied by the plants in the process of photosynthesis.

The hydrogen ion concentration of the solutions differed somewhat from culture to culture in individual sets, from set to set, and from series

to series. There were also slight variations in the hydrogen ion concentration of the same solution, depending upon the length of time the solution had been used in the culture. In the individual sets there were no differences in the pH values of the freshly renewed solutions, but towards the

TABLE V

EFFECT OF DIFFERENT CONCENTRATIONS OF CARBON DIOXIDE AND OXYGEN ON THE GROWTH OF POTAMOGETON PERFOLIATUS IN VARIOUS CULTURE MEDIA. JUNE 12 TO JULY 10, 1931

Content dissolved gases* (p.p.m.)		Addition to liquid	Sachs' nutrient solution diluted 1 to 30			Tap water		
CO_2	O_2		Total gain in length, cm.	Total length of roots produced, cm.	Increase in dry weight,** %	Total gain in length, cm.	Total length of roots produced, cm.	Increase in dry weight,** %
72		Soil	854	275	365	826	320	328
72		Sand	277	192	220	375	355	165
72		None	264	180	182	257	78	108
24		Soil	718	303	266	748	176	240
24		Sand	204	125	125	204	238	96
24		None	194	129	108	149	130	90
8		Soil	553	174	187	550	101	171
8		Sand	189	100	105	216	234	79
8		None	168	138	88	180	135	82
72	28	Soil	763	244	319	610	285	278
72	28	Sand	208	191	225	262	214	208
72	28	None	206	155	180	233	104	166
24	14	Soil	707	276	250	566	257	220
24	14	Sand	194	177	131	156	155	114
24	14	None	174	109	101	124	53	59
8	14	Soil	560	207	184	491	86	165
8	14	Sand	180	209	116	191	78	84
8	14	None	170	238	85	197	94	82
	28	Soil	261	175	117	280	60	105
	28	Sand	125	145	90	101	58	57
	28	None	122	279	81	98	42	48
	14	Soil	324	208	138	375	73	124
	14	Sand	161	249	102	238	143	75
	14	None	136	138	83	165	154	65
Ck.	Ck.	Soil	305	189	179	355	241	158
Ck.	Ck.	Sand	171	208	105	304	227	84
Ck.	Ck.	None	167	188	89	275	241	41

* Amounts of CO_2 and O_2 recorded in this column were kept as constant as possible throughout the day and night, respectively. In cultures for which no values are listed gases were not added, but the amounts in these varied with the medium, photosynthesis, respiration, etc. See text for explanation.

** Initial dry weight was calculated from comparable samples at the beginning of the experiment.

end of the experiment the pH of the solutions with a substratum of quartz sand sometimes varied as much as 0.5 towards the alkaline side. The extreme values found in the series in which Sachs' nutrient solution was used were pH 6.53 in cultures in which a concentration of 72 parts per million

TABLE VI

EFFECT OF DIFFERENT CONCENTRATIONS OF CARBON DIOXIDE AND OXYGEN ON THE GROWTH OF POTAMOGETON FOLIOSUS IN VARIOUS CULTURE MEDIA. JUNE 12 TO JULY 10, 1931

Content dissolved gases* (p.p.m.)		Addition to liquid	Sachs' nutrient solution diluted 1 to 30			Tap water		
CO_2	O_2		Total gain in length, cm.	Total length of roots produced, cm.	Increase in dry weight,** %	Total gain in length, cm.	Total length of roots produced, cm.	Increase in dry weight,** %
72		Soil	8137	4778	3524	1053	827	813
72		Sand	777	1441	344	265	279	214
72		None	506	728	340	150	154	155
24		Soil	3730	1928	1966	700	340	312
24		Sand	467	349	249	117	121	115
24		None	408	263	204	111	119	100
8		Soil	3661	2278	1551	570	295	309
8		Sand	371	235	231	98	83	76
8		None	295	199	190	80	75	66
72	28	Soil	5419	1370	2880	2078	1644	1473
72	28	Sand	651	960	342	295	251	191
72	28	None	439	627	242	282	225	186
24	14	Soil	3665	1448	1307	791	387	363
24	14	Sand	398	297	249	244	159	173
24	14	None	370	342	212	180	148	120
8	14	Soil	2304	856	1287	594	302	338
8	14	Sand	318	238	203	100	90	93
8	14	None	289	201	190	92	78	72
	28	Soil	1038	571	690	210	138	140
	28	Sand	260	223	113	61	62	58
	28	None	249	200	109	56	55	50
	14	Soil	1410	680	758	232	140	143
	14	Sand	277	257	130	79	70	64
	14	None	272	249	121	58	50	51
Ck.	Ck.	Soil	1450	569	820	543	290	302
Ck.	Ck.	Sand	308	228	177	90	72	67
Ck.	Ck.	None	324	229	173	64	68	56

* Amounts of CO_2 and O_2 recorded in this column were kept as constant as possible throughout the day and night, respectively. In cultures for which no values are listed gases were not added, but the amounts in these fluctuated with the medium, photosynthesis, respiration, etc. See text for explanation.

** Initial dry weight was calculated from comparable samples at the beginning of the experiment.

dissolved carbon dioxide was maintained and pH 8.22 in a solution with a quartz sand substratum in which only a concentration of 28 parts per million dissolved oxygen was maintained. The extreme values found in the tap water series were pH 6.2 in cultures in which a concentration of 72 parts per million dissolved carbon dioxide was maintained and pH 8.20 in cultures in which a concentration of 28 parts per million dissolved oxygen was maintained. The hydrogen ion concentration of most of the culture solutions, however, was found to be close to the point of neutrality.

The effect of different concentrations of dissolved carbon dioxide and oxygen on the growth of *Potamogeton perfoliatus* L. and *Potamogeton foliosus* Raf. cuttings in substrata of soil and quartz sand, or merely anchored without a substratum in dilute Sachs' solution and in tap water is shown in Tables V and VI. Here again, as in the experiments with *Naias flexilis* just described, the plants grew better in dilute Sachs' nutrient solution than in tap water; better in a soil substratum than in one of sand or in solutions without a substratum; and better in the cultures possessing the higher concentrations of carbon dioxide than in those to which no carbon dioxide was added. These experiments with *Potamogeton perfoliatus* and *Potamogeton foliosus* had the advantage over those with *Naias flexilis* in being carried on later in the season, from June 12 to July 10, when the periods of daylight were longer. Other conditions, however, were practically the same in all the experiments. As in the experiments with *Naias flexilis*, concentrations of dissolved carbon dioxide in those cultures for which no values are recorded in the first and second columns of the tables, and to which no carbon dioxide was added, were extremely low, especially in the solutions with sand substrata or without substrata. Dissolved oxygen, on the other hand, was present in all the cultures in quantities no less than that contained in tap water, but in those cultures to which carbon dioxide was added, and in which there was increased photosynthetic activity, the content sometimes during the day was as high as 17 parts per million. The hydrogen ion concentration of the solutions was within the range of pH values given for the experiments with *Naias flexilis*.

In the case of *Potamogeton perfoliatus* (Table V) the differences in growth between culture sets in the Sachs' nutrient solution series are not so great as in the tap water series. The optimum conditions for growth in both series are observed to be in soil cultures in which was maintained a content of 72 parts per million dissolved carbon dioxide. The addition of oxygen, in general, is seen to retard rather than to promote growth. The differences in growth between cultures in Sachs' nutrient solution and in tap water are relatively slight, compared with the growth of *Naias flexilis* and *Potamogeton foliosus*, except in some cases the plants anchored in Sachs' solution grew considerably better than those in comparable cultures of the tap water series. Heavy incrustations of calcium carbonate on the

leaves was a marked characteristic of *Potamogeton perfoliatus* plants anchored in the solutions or in solutions with a quartz sand substratum which was not observed in the case of the other species used in the experiments. Furthermore, the cuttings of *Potamogeton perfoliatus* exhibited another characteristic not observed in the other species; the original cuttings soon ceased to grow and usually died above the basal node. New shoots and runners were produced from this basal node. This factor makes an appreciable difference in the weights of the cultures grown for only a short period, as compared with the other species employed in the experiments, which did not possess this characteristic. There was a greater production of roots by cuttings of *Potamogeton perfoliatus* than by those of *Naias flexilis*, but there was no development of root hairs in the case of the plants anchored in the solutions.

With *Potamogeton foliosus* (Table VI) the differences in growth between plants grown in Sachs' nutrient solution and in tap water are quite pronounced, being more than twice as great in the Sachs' nutrient solution series. Sachs' nutrient solution had a more favorable effect upon the growth of *Potamogeton foliosus* than upon that of any other species used in the experiments. While the optimum conditions for growth in both series were found in the cultures in which the maximum concentrations of carbon dioxide were maintained, growth in the tap water series apparently was favored by the addition of oxygen at night when carbon dioxide was added during the day, which was not the case in the Sachs' nutrient solution series. The addition of oxygen to cultures receiving no carbon dioxide, however, tended to retard growth. In contrast to the other species used in the experiments, *Potamogeton foliosus* cuttings merely anchored in the solutions developed abundant roots, all clothed with root hairs, a characteristic not observed in the other species. Even the cuttings rooted in soil and quartz sand developed roots from the nodes above the substratum, and these roots were found to be liberally supplied with root hairs. The leaves of this plant are covered with a waxy cuticle upon which water does not spread.

The effect of nutrient solutions containing nitrogen in the form of nitrate or ammonium, or a combination of the two, with and without the addition of carbon dioxide, on the growth of *Potamogeton perfoliatus* and *Naias flexilis* cuttings in culture solutions with soil or quartz sand substrata, or anchored in the solutions without a substratum, is shown in Tables VII and VIII. In general, little difference is to be observed between the effects of nitrate and ammonium nitrogen in the case of either species. With *Potamogeton perfoliatus* cuttings, however, there were greater differences between the cultures in the individual sets than the percentages of increase in dry weight seem to indicate, because the original cuttings died and completely disintegrated above the basal nodes only in the soil cul-

tures. In the cultures with sand substrata and in those without a substratum the original cuttings soon ceased to grow, but remained green until the end of the experiment. Consequently, the dry weight totals for these cultures are augmented by the dry weight of the original cuttings. This factor, favoring the plants in sand and those anchored in the solu-

TABLE VII

EFFECT OF NITRATE AND AMMONIUM NITROGEN, WITH AND WITHOUT THE ADDITION OF CARBON DIOXIDE, ON THE GROWTH OF POTAMOGETON PERFOLIATUS IN VARIOUS MEDIA. DECEMBER 7, 1931 TO JANUARY 4, 1932.*

Form of nitrogen	Content dissolved CO_2** (p.p.m.)	Addition to liquid	Total gain in length, cm.	Total length of roots produced, cm.	Increase in dry weight,† %
Nitrate	72	Soil	839	299	370
	72	Sand	285	275	242
	72	None	283	196	193
		Soil	294	199	198
		Sand	254	195	162
		None	180	204	143
Ammonium	72	Soil	783	255	334
	72	Sand	282	236	223
	72	None	221	230	180
		Soil	270	113	175
		Sand	158	166	144
		None	157	159	138
Nitrate and ammonium	72	Soil	809	280	359
	72	Sand	286	250	231
	72	None	225	177	184
		Soil	290	150	190
		Sand	181	179	151
		None	169	174	140

* Daylight supplemented by 4 hours of artificial light from four 1500 watt lamps.

** Amounts of CO_2 recorded in this column were kept as constant as possible throughout the day. In cultures for which no CO_2 values are listed no gas was added, the amounts varying with the medium, photosynthesis, respiration, etc. See text for explanation.

† Initial dry weight was calculated from comparable samples at the beginning of the experiment.

tions, causes the difference in percentage increase in dry weight between these and the plants in the soil cultures to appear smaller than that which actually existed.

Growth of *Potamogeton perfoliatus* cuttings was slightly superior in solutions containing nitrate and in those containing both nitrate and ammonium nitrogen to that in solutions possessing nitrogen only in the form of ammonium. With cuttings of *Naias flexilis*, however, just the reverse was true, as growth was better in the cultures containing nitrogen in the

form of ammonium, but the rooting of anchored plants was superior in the solutions containing nitrate.

The addition of carbon dioxide increased the growth of plants in all cultures. Oxygen was not added to the cultures, because the results of previous experiments had indicated that the quantity of dissolved oxygen present in the solutions, even without the oxygen supplied to the solutions by the plants in the process of photosynthesis, was more than sufficient to meet the respiratory requirements of the plants.

TABLE VIII

EFFECT OF NITRATE AND AMMONIUM NITROGEN, WITH AND WITHOUT THE ADDITION OF CARBON DIOXIDE, ON THE GROWTH OF NAIAS FLEXILIS IN VARIOUS MEDIA. DECEMBER 7, 1931 TO JANUARY 4, 1932.*

Form of nitrogen	Content dissolved CO_2** (p.p.m.)	Addition to liquid	Total gain in length, cm.	Total length of roots produced, cm.	Increase in dry weight,† %
Nitrate	72	Soil	917	149	248
	72	Sand	165	106	143
	72	None	154	100	138
		Soil	397	109	148
		Sand	145	96	143
		None	141	110	99
Ammonium	72	Soil	980	167	273
	72	Sand	244	125	191
	72	None	158	112	146
		Soil	400	126	153
		Sand	202	123	134
		None	191	10	121
Nitrate and ammonium	72	Soil	926	122	255
	72	Sand	218	125	169
	72	None	168	15	140
		Soil	396	115	147
		Sand	197	104	109
		None	187	12	86

* Daylight supplemented by 4 hours of artificial light from four 1500 watt lamps.

** Amounts of CO_2 recorded in this column were kept as constant as possible throughout the day. In cultures for which no CO_2 values are listed no gas was added, the amounts varying with the medium, photosynthesis, respiration, etc. See text for explanation.

† Initial dry weight was calculated from comparable samples at the beginning of the experiment.

By frequent changes of the culture solutions their hydrogen ion concentration was kept within a narrower range in this than in previous experiments in which nutrient solutions were employed. All pH determinations were made by the glass electrode method. The standard Sachs' solution before dilution had a value of pH 6.41, but after dilution with tap

water in the proportion of one volume of the solution to 30 volumes of water the value was pH 7.23, which was lowered to a minimum of pH 6.55 in cultures, just before renewal of the solutions, to which carbon dioxide had been added. The values for cultures to which carbon dioxide was not added ranged from pH 7.23 to pH 7.62, depending upon the length of time the solutions had been used in the cultures. The solution in which ammonium sulphate and potassium sulphate were substituted for potassium nitrate had a value of pH 6.20 before and pH 7.18 after dilution with tap water. The lowest value for this solution was pH 6.51 in cultures to which carbon dioxide was added. In the other cultures the values ranged from pH 7.18 to pH 6.84. A mixture of the two standard solutions had a value of pH 6.32 before and pH 7.21 after dilution with tap water. In cultures receiving carbon dioxide the lowest value determined for this mixed solution was pH 6.50, and the highest value in cultures not receiving carbon dioxide was pH 7.55.

In an additional experiment, conducted for the purpose of determining the rooting response of *Naias flexilis* cuttings, the plants grew better in shaded cultures in which dilute Sachs' solution was used. Tap water cultures exposed to full light was the least favorable of the four experimental conditions. No difference was found in the rooting response of the cuttings in individual cultures. Plants merely anchored or tied to a smooth surface in the solutions developed just as many roots as those tied to a sand surface. Contact of the plants with a rough or a smooth surface did not stimulate root development. The development of roots was better in shaded Sachs' nutrient solution cultures, but it was also good in the same cultures exposed to full light. In exposed cultures, however, both growth and root development were less than in the shaded cultures. Light did not retard root development any more than it retarded the growth of the entire plant. In general, root development was found to be an expression of the degree of plant growth.

DISCUSSION

Salinity. The results reported in preceding sections of this paper demonstrate clearly that the salt content of the water in Back Bay, Virginia, during the years from 1925 to 1930, inclusive, was not directly responsible for the destruction of the growths of the dominant submerged seed plants. *Potamogeton pectinatus* L., the most abundant plant in the region, and *Potamogeton perfoliatus* L., another important local duck-food plant, have been shown in experiments to thrive in concentrations of sea water having a higher total content of sodium chloride than the maximum recorded for the water of Back Bay. With the possible exception of the extreme northern part of Currituck Sound, none of the samples of water in the entire region during the period of the investigation was found with a salt content

too high for the growth of these two plants. While the experiments with *Vallisneria spiralis* L. are inconclusive because of the time of year at which they were conducted, the results indicate nevertheless that the salt content of Back Bay at no time during the investigation was too great for the growth of this plant. All these species are known to grow in such brackish waters as Barnegat Bay, New Jersey, with a higher content of total salts than that found in Back Bay and most of Currituck Sound. While *Ruppia maritima* L. was not used in the experiments because of difficulties encountered in overcoming its susceptibility to growths of algae in stock cultures kept in the greenhouses, Osterhout (57) succeeded in growing this species in undiluted sea water. The results of the present experiments confirm Osterhout's conclusion that, for the aquatic plants that he used, sea water is a "physiologically balanced solution." Kearney (39) has expressed the opinion that "it is usually not so much the chemical composition of the soil solution as its concentration and the resulting osmotic pressure which affects vegetation." He excepted sodium carbonate, since this salt is much more toxic than the other sodium salts. The osmotic effect of a solution, however, would seem to depend to a great extent on the permeability of the protoplasmic membranes of the plant cells to the solutes of the external solution. True (94) found, for example, that much higher osmotic pressures of sea water than of cane sugar solutions were required to plasmolyze the cells of certain algae. He determined that an osmotic pressure of 11.7 atmospheres in sea water was required to produce the plasmolytic effect of 6.6 atmospheres in a cane sugar solution, and he attributed the difference as being due probably to the penetration of the sodium chloride of sea water into the plant cells. The results of the present experiments show that the growth of the experimental plants was checked in concentrations of sea water still too weak to plasmolyze their cells. This inhibition may have been due to a decrease in photosynthetic activity induced by sodium chloride, as Jacobi (34) observed that a 0.29 per cent concentration of this salt retarded photosynthesis in *Elodea canadensis* about 50 per cent and increased respiration from 25 to 50 per cent at 22° C. This depressive effect of salt on the process of photosynthesis was later confirmed by Treboux (93), who adopted the view that salts, such as sodium chloride and potassium nitrate, decrease assimilation activities by altering the water relations within the cells. Walter (96, 97) also observed that the rate of assimilation in *Elodea canadensis* was closely associated with the osmotic relation of the cell sap to the external solution, but the withdrawal of moderate amounts of water from the cells by osmotically active substances in the external solutions was found not to influence greatly respiration.

While the salinity of the waters of Back Bay and Currituck Sound cannot be held directly responsible for the destruction of the aquatic seed

plants in the region, the fluctuating salt content of these waters is to be considered as a measure of the change in the natural conditions brought about by opening the Albemarle and Chesapeake Canal. This canal has been shown to be the main source of salt water entering Back Bay and Currituck Sound. Other possible sources such as sea water entering these waters by flowing across or seeping through the barrier beach can be disregarded so far as the destruction of aquatic plants is concerned. These conclusions were reached also by the U. S. Army Engineers (36), who found that the salt content of these inland waters decreased as the distance south from Norfolk increased to the southern part of Currituck Sound where the water was relatively fresh, and that other possible sources of salt water were insignificant. The quantities of sea water which flowed in time of storms over low places in the barrier beach into Back Bay and Currituck Sound during the course of this investigation were more beneficial than injurious to the growth of aquatic plants, since this sea water, in contrast to that entering from Norfolk Harbor, was found to precipitate the suspended matter, to clear the water, and not to increase the salt content of the water higher than that having more than a stimulating effect upon the growth of the dominant aquatic seed plants. On the other hand, salt water penetrating these inland waters from Chesapeake Bay and Norfolk Harbor by way of the Albemarle and Chesapeake Canal had an essentially different effect upon the plant life. Clear water was replaced by water frequently so turbid that total darkness occurred at depths of three feet, by water too muddy and polluted to support the growth of aquatic seed plants. Another important result of the changes this water brought about in the natural conditions was the disappearance of the fresh-water fishes, and the appearance of hydroids and organisms usually associated with polluted and sewage contaminated waters. The increasing and fluctuating salt content of the water is, therefore, one of the best measures of the quantity of turbid, polluted salt water flowing southward into Back Bay and Currituck Sound. It has been suggested by Jewett (36) that deficiencies in the amount of rainfall might account for some of the fluctuations in the salt content of these waters. This, however, was found not to be the case. The direction, velocity, and duration of the winds have been shown to be responsible for the fluctuations in salinity by causing northward and southward flows of water through the Albemarle and Chesapeake Canal. The fluctuations in the salt content is correlated closely with the changes in the turbidity of the waters of Back Bay and Currituck Sound.

Light. The results of field determinations and laboratory experiments show conclusively that the turbidity of the waters of Back Bay and Currituck Sound has probably been the chief factor responsible for the destruction of the submerged seed plants. *Potamogeton pectinatus* L. was found by experimentation to require for the support of growth more than 3.5 per

cent of the total solar energy. This is a greater quantity of light than that usually measured on the bottom of Back Bay or Currituck Sound, for in these waters this minimum light requirement for the support of the growth of *Potamogeton pectinatus* was found frequently at depths of water less than three feet. During the greater part of each season scarcely sufficient light for photosynthetic activity was transmitted by the clearest water in the region.

The deep layer of sludge on the bottom of Currituck Sound and Back Bay is responsible for the excessive turbidity of the water. While the Albemarle and Chesapeake Canal undoubtedly has been the main source of this sludge, it seems unlikely that the water will be cleared up even by the installation of guard locks in this canal. In the absence of "bottom-cover" plants wind and wave action keep the finely divided particles of mud in an almost constant suspension; even in water fairly still these particles settle out very slowly. It is believed that only the reestablishment of a "bottom-cover" with growths of such plants as *Chara* and *Nitella* will prevent the riling of the water and bring about its normal clearness.

Wolkoff (102), in 1866, apparently was the first worker to investigate the effect of light intensity on the rate of photosynthesis in aquatic plants. Working with *Ceratophyllum demersum* and *Potamogeton natans*, he discovered that the rate of photosynthesis was proportional to the light intensity for low intensities, but that the rate was not proportional in strong light. Three years later, van Tieghem (91, 92) arrived at the same conclusion. Prillieux (64, 65), in 1869, observed that yellow and orange light was more effective than red, blue, and violet on the rate of photosynthesis in *Potamogeton perfoliatus*, but he gave no energy measurements in these regions. He also determined that *Elodea canadensis* needed only one-half or less of full sunlight to carry on photosynthetic activity and that the plant in the process could use artificial light instead of sunlight. Reinke (67, 68), experimenting with *Elodea canadensis* in water, which was charged with a small amount of carbon dioxide, at temperatures of 20° to 28° C., observed that the rate of photosynthesis was not increased in light intensities stronger than one-half sunlight. The long level part of his curve was regarded as a prolonged optimal effect. Pantanelli (59) found the optimum light intensity at one-fourth full sunlight for photosynthetic activity of plants growing in spring water. This optimum was found to shift towards stronger light with increases and towards weaker light with decreases in the carbon dioxide supply. Drops in his curves towards stronger light intensities were attributed to plastid fatigue. Pfeffer (60, p. 324) calls attention to Reinke's work and suggests the possibility of deficient carbon dioxide being a limiting factor. Blackman and Smith (12) give a different interpretation of Pantanelli's results, introducing a time factor, and make the same criticism of Reinke's results as that made by Pfeffer. As a result of experiments with

Elodea and *Fontinalis*, Blackman and Smith (12, p. 411) came to the conclusion that the relation between assimilation and the chief environmental factors of carbon dioxide supply, light intensity, and temperature is such that the magnitude of the function of assimilation "in every combination of these factors is determined by one or other of them acting as a limiting factor."

The method of measuring light intensity on land by the Shirley thermoelectric radiometer has been subjected to criticism because the instrument records the infra-red region, which constitutes more than 50 per cent of the total solar energy. The infra-red is not only of no use to the plant in the process of photosynthesis but is also found to vary greatly according to the time of day or season. This criticism, however, cannot be applied to measurements of light intensity made under water, because the water acts as a selective filter to absorb practically all the infra-red in the first inch. In water, therefore, the instrument records not only total radiant energy at various depths but also measures only the visible region, which is the portion of radiant energy available to aquatic plants. The most effective region of this available radiant energy on the photosynthetic activity of *Elodea canadensis* was found by Reinke (68) to be in the red at wave length 680 mμ, between Fraunhofer lines B and C. Reinke also observed that effectiveness of the regions of the available radiant energy falls off quickly from this point towards the infra-red and slowly towards the ultra-violet.

The particles carried by water in suspension are nonselective in the absorption of light. This fact was discovered by Pietenpol (61) in 1918 and confirmed in the present investigation in water samples from Back Bay, Virginia, by determinations made with a König-Martens spectrophotometer. Different types of water, however, differ in their capacities to absorb light. Magnin (52) in an investigation of the lakes of Jura found in some lakes that 60 to 90 per cent of the radiant energy was absorbed by the first meter of water. Birge and Juday (10) state that in highly colored or turbid lakes the zone of photosynthesis may be only two to three meters in thickness, but that in clear lakes it may extend to ten meters or more. Birge (9) writes that the transparency of lake water is affected by the turbidity of suspended matter and by stain from material such as peat. His observations made on more than 25 lakes show that not more than 20 per cent of the light is found at one meter depths and that the amount is usually much less, sometimes as low as 2 to 2.5 per cent. His observations show also that not less than 30 per cent of the energy present at a depth of one meter is absorbed by the stratum of water between one and two meters. The rate of absorption per meter was found to be substantially the same in subjacent meters as it was between one and two meters. Birge states that the absence of correlation between transparency of water and the rate

of energy absorption is not uncommon, because stained water may be more transparent than turbid water and in the former the rate of energy absorption may be "relatively or absolutely greater." It would seem, therefore, that no strict rule can be applied to the absorption of light by natural waters. It is surprising, however, that plants have existed even in the clearest waters of the Back Bay and Currituck Sound region because of the great absorption of light by the water.

While the light requirements have been determined for only one species, *Potamogeton pectinatus*, it is believed that the growth of other submerged angiosperms is prohibited by such turbidities as exist in Back Bay and Currituck Sound. Montfort (55) describes aquatic "sun and shade" plants and states that heterogeneous ecological types of light adjustment have been developed in water as on land. If such be the case, there may be species of submerged angiosperms with the capacity to carry on photosynthetic activity on less light than that required by *Potamogeton pectinatus*. In this connection, Kostytschew and Soldatenkow (43) observed that the daily course of photosynthesis in aquatic plants was not uniform but that it reached its maximum rate in the morning and dropped in the afternoon when light and temperature conditions were very favorable for the process. They noted specific differences in the photosynthetic rate and activity among the species studied. Despite possible differences in the light requirements of different species or ecological types, however, it seems improbable that any green plant could thrive in water so turbid as that existing in Back Bay and Currituck Sound during the period of this investigation. Plants existing in the clearest water in shallow, relatively isolated waters of the region present the etiolated appearance and the anatomical characteristics of the shaded specimens described by Möbius (54), who found that shaded or darkened plants developed extremely long internodes and few or no roots. A pronounced characteristic of the *Potamogeton pectinatus* plants still found growing in the clearer waters of North Bay and Back Bay is the small production by the underground stems of starchy tubers, both as regards size and quantity; tubers obtained from clear fresh waters generally have been found to be many times as large.

Dissolved gases. While the low content of dissolved oxygen found in the waters of Back Bay and Currituck Sound may be the best indication of the degree of pollution, no evidence has been found to show that this is a limiting factor in the growth of submerged seed plants in these waters. The works of Birge and Juday (10, 11), Richardson (69, 70, 71), Thompson (87, 88), and Wiebe (100) indicate that such contents of dissolved oxygen as exist in these waters are too low to support life in some fresh-water fishes. Thompson (87) observed an appreciable number of fish species only in water containing four or more parts per million and a large number of species only in water possessing nine or more parts per million. Moreover,

the relatively high temperature of the water of Back Bay and Currituck Sound during the summer probably reduces the value of the content of dissolved oxygen present, as Ruttner (74) finds that oxygen consumption by aquatic organisms is almost doubled by a rise in temperature of 10° C., and that the same amount of oxygen is worth about twice as much for respiration at 5° as at 15° C. The low content of dissolved oxygen, with its respiration value thus reduced, may at least partly account for changes in the local fauna which probably may have influenced somewhat the disappearance of aquatic seed plants. Fish that tend naturally to keep down the numbers of organisms parasitic and injurious to plant life (especially the hydroids which are food competitors of fish), have practically disappeared from these waters. Herbivorous species, such as the carp which Thompson (88) describes as quite destructive to aquatic seed plants, are, on the other hand, able to withstand a content of dissolved oxygen as low as 2.5 parts per million. This type of fish, which includes the carp, mullet, and mud shad, is now the most abundant in Back Bay and Currituck Sound, and during the present investigation was found very destructive to experimental plantings in the small ponds. Considering the size of the water area of the region, however, the favorable or unfavorable influence of fish on the growth of submerged seed plants is probably only slight. The low content of dissolved oxygen in these waters and the disappearance of certain species of fish are not considered as factors limiting the growth of aquatic seed plants, but rather as indicators of the disturbance of a balance in nature and of the changes in the natural conditions which have brought about the loss of submerged duck-food plants to the region. While Arber (4, p. 255) states that "as far as hydrophytes are concerned, oxygen is a rare and precious commodity," the results of the present experiments and determinations in nature seem to show that, under favorable conditions of light, carbon dioxide supply, and temperature, submerged plants in the presence of light produce in the process of photosynthesis more oxygen than is necessary for their own requirements. As early as 1850, Cloez and Gratiolet (23) observed that submerged plants in the presence of sunlight absorb carbon dioxide and give off oxygen. They described the evolution of oxygen as beginning at 15° and reaching its maximum at 30° C. The gas given off during the process of photosynthesis by submerged plants, however, is not entirely oxygen. Cloez (22), in 1863, determined that the gas evolved by *Potamogeton perfoliatus* plants exposed to light is a mixture of oxygen and nitrogen. A few years later, van Tiegham (89, 90, 91) described the gas bubbling from wounds in the green shoots of submerged plants as being composed of about 90 per cent oxygen and 10 per cent nitrogen. The physics of gaseous exchange in water plants and the proportions of atmospheric gases dissolved in water were worked out more accurately by Devaux (26) in 1889. He found that at 15° C. 33.98 per cent of dissolved

air is oxygen. Very recently, Górski (28) reports that the bubbles of gas set free in the course of photosynthesis by *Elodea canadensis* and *Potamogeton lucens* consist of a mixture of oxygen (21 to 50 per cent), nitrogen (50 to 80 per cent), and carbon dioxide (2 per cent). The percentage of oxygen was found by him to increase with the intensity of light, and the quantity of oxygen contained in the bubbles to be only approximately proportional to the rate at which the bubbles were produced, being relatively greater for greater intensities of photosynthesis. Regardless of the proportion of oxygen produced in the process of photosynthesis, however, the content of dissolved oxygen in water supporting growths of submerged seed plants is more often found unusually high. Birge and Juday (10) frequently found such water more than saturated with oxygen. Furthermore, the numerous large lacunae present in most species of submerged angiosperms undoubtedly provide for the retention within the plant of appreciable supplies of oxygen for use in darkness. It was observed in the laboratory cultures during the present experiments that very little dissolved oxygen was withdrawn at night from the solutions by the respiration of the plants, and that the addition of oxygen to solutions containing growing plants served no purpose.

The question of the influence of a carbon dioxide supply on the growth of aquatic seed plants, however, is essentially different from that of oxygen. Arber (4, p. 253) has stated that all observers appear to agree that the water of lakes and streams, under natural conditions, is supersaturated with carbon dioxide. This is certainly not true of free carbon dioxide, which fact has been well stated by Whipple (99, p. 296), who writes: "Streams undefiled by any other than Nature's pollution will contain at most only a few parts per million of carbon dioxide." Waters supporting the growth of submerged plants usually react alkaline to phenophthalein, as Birge and Juday (10, 11) observed, and seldom contain more than a trace of free carbon dioxide. In nature, however, carbon dioxide exists in three different states: fixed, as carbonates; half-bound, as bicarbonates; and as free carbon dioxide. Fixed carbon dioxide usually exists in the form of the normal carbonates of calcium and magnesium, which are only slightly soluble in pure water, and the half-bound, ordinarily as the bicarbonates of these elements, which readily pass into solution. There seems to be a certain balance in natural waters between these three different states of carbon dioxide, which has been described by Birge and Juday (11, p. 583) somewhat as follows: Rainwater, absorbing carbon dioxide from the air, and drainage water, obtaining carbon dioxide from decomposing organic matter, come in contact with carbonates and freely convert them into bicarbonates, which are soluble in water and which thus serve as a very important source of carbon dioxide for the photosynthetic activities of aquatic angiosperms. Birge and Juday (11) assert that from four-fifths to five-sixths

of the bicarbonate content of water may be consumed in the process of photosynthesis, but that none of the fixed carbon dioxide is available. Ruttner (72, 73) maintains that aquatic angiosperms depend chiefly on calcium bicarbonate for a supply of carbon dioxide and that the assumption of Nathansohn (56), that aquatic plants do not effect the decomposition of carbonates, is not justified.

While both carbon dioxide solutions and bicarbonate solutions have been freely utilized by numerous workers as methods of supplying carbon dioxide in physiological experiments dealing with the relation between the concentration of carbon dioxide and the rate of photosynthesis in aquatic plants, the form of carbon dioxide used in the photosynthetic activities of aquatic plants is still a matter of controversy. "For these methods to be directly comparable with one another," James (35, p. 2) states, "it must be assumed that only carbon dioxide present in the free form is assimilable by the plant, and that HCO_3 ions and undissociated salts ($NaHCO_3$, etc.) are not available." This assumption has been supported by Benecke (8), Nathansohn (56), and Wilmott (101), but opposed by Angelstein (3), Arens (5), Osterhout and Haas (58), and Ruttner (72, 73). The view of the former group is that dilute solutions of bicarbonates give initial carbon dioxide diffusion effects and generally behave as solutions of carbonic acids, while the latter, in general, holds that aquatic plants, such as *Elodea*, have the power to split bicarbonates. Among the latter group, Arens (5) recently concluded from experimental results that the incrustation of calcium carbonate on the upper surface of the leaves of many aquatic plants, previously described among others, by Pringsheim (66) and earlier by Cloez and Gratiolet (23), is caused by calcium bicarbonate entering the underside of the leaf as an entire molecule, losing its carbon dioxide in the assimilating tissue, and leaving the leaf on the upper surface as free calcium oxide or hydroxide molecules, which unite with carbon dioxide in the water and become precipitated on the upper surface of the leaf as calcium carbonate. Regardless of the form in which carbon dioxide may be utilized by the plant, however, both carbon dioxide solution and a dilute solution of a bicarbonate appear to have been employed successfully as sources of carbon dioxide in the physiological experiments of various workers, although the results of the two methods sometimes appear to have little in common. For example, in experiments to determine the relation between carbon dioxide concentration and the rate of photosynthesis in aquatic plants, Blackman and Smith (12), using flowing solutions of carbon dioxide, and Harder (30), employing bicarbonate solutions which remained at rest, obtained essentially different results with varying concentrations of the solutions. James (35) reported the two results readily repeatable, and came to the conclusion that much of the apparent disagreement was due to differences in the experimental conditions employed. He found that the

rates of assimilation in solutions of carbon dioxide and sodium bicarbonate with equal partial pressures of free carbon dioxide were practically equal. The rate of flow of dilute bicarbonate solutions was observed to have no effect on the rate of photosynthesis, a result quite the opposite from that obtained with solutions of only carbon dioxide. In low light intensities with bicarbonate solutions the rate of photosynthesis about equalled the highest obtained with the flowing carbon dioxide solutions, but in high light intensities the rate in the bicarbonate solutions greatly surpassed that caused by a rapid flow of a corresponding solution of carbon dioxide. From these results, it appeared to James that the "buffer action" of the bicarbonate is more effective in maintaining a potential supply of carbon dioxide near the surface of the chloroplasts than any practicable movement of the solutions, and may reduce diffusion resistance to a low value. James further concludes (35, p. 38): "Since under favorable conditions a pure carbon dioxide solution could bring about the same rate of reaction as a bicarbonate solution the experiments provide strong evidence that only the free carbon dioxide is normally used in assimilation."

It is to be pointed out, however, that the concentrations of carbon dioxide and bicarbonate solutions used by most of these investigators in their work on photosynthesis have been higher than concentrations encountered by plants in nature. This fact seems to have been recognized by Osterhout and Haas (58), who studied plants in natural sea water, rather than in solutions containing unusual amounts of carbon dioxide. Furthermore, the experiments of all these workers were carried on over very short periods of time, as many of them lasted only a few minutes. No attempts seem to have been made to grow aquatic plants for an appreciable length of time under their experimental conditions. In some preliminary experiments connected with the present investigation, it was found that such concentrations of carbon dioxide as those used by Blackman and Smith (12), Reinke (67, 68), and others were fatal to the experimental plants within about a week.

The tap water used in the cultures of the present experiments, as the tap water reported by Brown (16), was found to contain insufficient carbon dioxide to support growth in the experimental plants. The concentrations of carbon dioxide maintained in the solutions, however, were only arbitrarily chosen as 8, 24, and 72 parts per million. An additional multiple of these concentrations, 216 parts per million, was not employed after the results of preliminary experiments showed it to be toxic to the experimental plants, although the final results of the experiments indicate that higher concentrations than 72 parts per million of carbon dioxide might have been employed advantageously, especially in the case of the nutrient solution series. In general, the addition of carbon dioxide to the cultures stimulated the growth of the plants, and the increase in growth was roughly

proportional to the concentration of carbon dioxide maintained in the culture solutions. In this respect the observations of Brown (16) are confirmed. The experimental results, however, do not confirm his conclusion that bubbling carbon dioxide through tap water cultures several times a day will eliminate differences in growth between plants rooted in soil and those merely anchored in tap water, for in every condition of the experiments plants grew better when rooted in a soil substratum. Furthermore, the experimental results and the field observations made during the course of the present investigation indicate that carbon dioxide supply has not been a factor limiting the growth of submerged angiosperms in Back Bay and Currituck Sound.

Nutrient solutions and substrata. In nature, the plants employed in the experiments reported in this paper are found usually in neutral or alkaline waters. For this reason Sachs' nutrient solution, which is more nearly neutral in reaction than other nutrient solutions commonly employed, was chosen for the experiments. Pond (63) selected this nutrient solution for the same reason and because it was believed that algae would not live in it. He used the standard solution in his experiments with submerged plants and found it the least favorable of several conditions. Snell (83) employed various dilutions and strengths of Crone's solution, apparently with little success. Brown (16), in his work with *Elodea*, selected Knop's solution and tried concentrations from one-half to four times the strength of the standard solution, obtaining the best results with twice standard strength. He reports an increase in growth of *Elodea* in this solution 48.2 per cent greater than the growth of plants in comparable tap water cultures. He considered this increase small, however, when compared with the increase in growth produced by variations in the amount of carbon dioxide in tap water.

The hydrogen ion concentrations of the nutrient solutions most commonly used have been determined recently by Åslander (6). He reports values of pH 3.65 for Knop's solution, pH 4.49 for Crone's solution, and pH 6.46 for Sachs' solution. His experimental results indicate that plants are able to grow in solutions as acid as Knop's only when the concentrations are relatively high. In experiments in which diluted solutions were used and the original pH value maintained the growth of the plants declined with dilution. The fact that plants grew well in nutrient solutions containing a relatively high salt content and acid in reaction led Åslander to believe that the salts in some way counteracted the injurious action of the hydrogen ions. He found also that plants grew well in a very dilute solution when it was nearly neutral in reaction. His findings and conclusions probably explain Brown's (16) surprising results with twice the standard strength of Knop's solution.

In the present investigation preliminary experiments were conducted with Sachs' solution full strength, one-tenth, one-thirtieth, and one-nine-

tieth standard strength. The first two concentrations were found to be too strong, as the plants in them died within two weeks, but the last two were found about equally good. These results indicate that the concentration of Sachs' solution employed by Pond (63) was much too strong for the growth of his experimental plants. Furthermore, in cultures of the present experiments containing diluted Sachs' solution, the growth of algae was controlled only with difficulty. In such cultures not receiving carbon dioxide, however, algae ceased to thrive when growth of the experimental plants reached the surface of the culture solution. This check in the growth of algae can not have been caused by a deficiency of mineral nutrients, because the solutions were renewed frequently, but by an insufficient carbon dioxide supply. This is contrary to the view expressed by Kofoid (40), who attributed the scarcity of plankton in lakes containing submerged vegetation to a number of causes, but chiefly to the removal of a great part of the available mineral nutrients by the submerged seed plants. It is likely, however, as Whipple (99, p. 296) suggests, that the removal of free carbon dioxide is a more important factor, for algae persist in cultures containing aquatic seed plants only as long as there is an excess of carbon dioxide present.

While Sachs' solution is less favorable as a nutrient medium than soil solutions, it was found to increase the growth of all species used in the experiments, the greatest increases being noted in the case of *Potamogeton foliosus* Raf. This plant differs from other species used in the experiments in that the leaves have a lustrous, wax-like surface and in that the plants produce abundant root hairs on the roots not developed in a substratum. The leaves, although all are submerged, shed water. This fact is due, according to Lundström (45), to the production in the epidermal cells of "oil plastids" which secrete droplets of oil. Lundström expresses the opinion that this oily substance serves as a means of protecting the plant from aquatic insects and of preventing the diffusion of substances, such as glucose, from the plant cell into the surrounding water. The anatomical and physiological characteristics of *Potamogeton foliosus* suggest that the absorption of mineral nutrients by this plant may be entirely through the roots.

No appreciable differences were observed between the growth of *Potamogeton perfoliatus* and *Naias flexilis* in Sachs' nutrient solution and that in a similar nutrient solution in which ammonium nitrogen was substituted for the nitrate in Sachs' solution, or in a mixture of the two solutions. *Potamogeton perfoliatus* grew slightly better in solutions containing nitrate and *Naias flexilis* in those containing ammonium nitrogen, but the differences are rather insignificant. Pirschle (62), in his studies on nitrate and ammonium as sources of nitrogen for higher plants at constant hydrogen ion concentrations, concluded that *Elodea canadensis* grew better in a nu-

trient solution containing ammonium nitrogen than in one containing nitrate at an optimum hydrogen ion concentration of pH 5.0. He also obtained good results with the ammonium at pH 4.0 and at pH 6.0. His data show, however, that growth in the solutions containing nitrate nitrogen at pH 5.0 is nearly as good, on the basis of both fresh and dry weight, as that in the solutions containing ammonium nitrogen, and a second nitrate optimum at pH 9.0 shows a better growth of the plants than that in the solutions containing ammonium nitrogen at pH 5.0. In the present experiments the hydrogen-ion concentrations of the solutions were kept within a relatively narrow range and no conclusions can be drawn concerning the effect of nitrate and ammonium nitrogen on the growth of the experimental plants at high and low pH values.

In every condition of the experiments, the plants grew better when rooted in a soil substratum. In this respect, the results agree with those of Pond (63), but disagree with those of Brown (16). While growth in cultures containing diluted Sachs' solution and a quartz sand substratum, and to which carbon dioxide was added, was relatively good when compared with that in soil and tap water cultures without additions of carbon dioxide, no condition was found to replace the beneficial effect of a soil substratum. The plants, however, grew well and remained green and healthy when rooted in quartz sand and Sachs' solution, and in most cases (if roots were developed) when merely anchored in diluted Sachs' solution to which carbon dioxide was added. In this respect the results do not confirm the conclusion of Pond (63), that submerged plants cannot survive a single season if denied a substratum of soil. The rooted condition in a soil substratum may be necessary for the growth of some species of submerged plants, as Pond concludes, or a particular type of soil may be required for some particular species, as reported by Stockmayer (85), but the species employed in the present experiments thrived in nutrient solutions without soil, especially in those to which carbon dioxide was supplied. This indicates, as Brown (16) suggests, that a deficient carbon dioxide supply may have been a limiting factor in Pond's experiments. Furthermore, the plants generally grew better and produced more roots in a quartz sand substratum than in solutions without a substratum. This superior growth in a quartz sand substratum may be due, as Pond (63) suggests, to some alteration in the quality of the solution by the sand particles. The development of roots and root hairs was usually greater in a sand than in a soil substratum. Additional results, however, indicate that the production of roots is not initiated by mere contact of the plant with a sand surface nor by the effect of light or darkness. In general, the rooted condition was found to be an expression of the degree of vigorousness of growth in the entire plant, and its value to differ with the species. For example, the development of roots and root hairs in *Potamogeton foliosus* was found superior to that in the

other species studied. This superiority apparently is not only associated with a more vigorous growth but may be considered as an indication that the roots of this plant are more important as organs of absorption than those of the other species, especially those of *Naias flexilis*. In the latter plant there is a relatively small development of roots, even in plants making measurable quantities of growth, which probably indicates that at least some absorption of mineral nutrients by this plant must occur through the leaves. The root system and the anatomical and physiological characteristics of *Potamogeton foliosus*, however, would seem to make untenable the view of Brown (16) that the roots of submerged plants serve only as organs of attachment, although the exact part that the roots of this plant take in the absorption of mineral nutrients remains to be determined.

Since the waters involved in the present investigation were found to be practically neutral in reaction, the effect of varying hydrogen ion concentrations on the growth of aquatic plants was not determined. This does not imply, however, that the relation between hydrogen ion concentration and plant growth is considered unimportant. While the species studied in the present experiments are widespread in distribution, they are reported usually as growing in waters almost neutral or in those alkaline in reaction. The effect of hydrogen ion concentration on their growth and distribution, therefore, should form an interesting study.

The species of plants included in the present studies form a part of a comparatively small group of angiosperms serving as the main source of food for wild ducks, geese, swan, and other wild fowl. The economic importance of these plants has been only recently appreciated in this country after the disappearance of great quantities of the plants from the principal feeding regions of the migratory game birds, caused by the drainage or the pollution of immense water areas. In addition to their value as food for wild fowl, these plants furnish protection to young fish and supply available food material to other aquatic organisms. The quantity of life which waters such as Back Bay and Currituck Sound can support is in general a function of the amount of seed plants which they can produce. Many problems connected with the physiology of aquatic angiosperms, their distribution, propagation, and colonization in new areas, as well as with the restocking and reseeding of depleted waters, remain as yet unsolved. In an effort to correct the damages to the flora of Back Bay and Currituck Sound, the U. S. Government recently (June, 1932) completed the construction of locks in the Albemarle and Chesapeake Canal. Changes probably will occur in the conditions and in the flora of these waters after the restoration of the locks. An intensive study should be made of these changes not only to increase our knowledge of the biology of such waters but also to furnish data for recommendations regarding the economic

preservation of areas that are now or may be in the future subject to similar dangers.

SUMMARY

Ecological studies were made of the conditions responsible for the destruction of the aquatic seed plants, which formerly were an important source of food for wild fowl, in the waters of Back Bay in Virginia and Currituck Sound in North Carolina, a region approximately 200 square miles in area and famous as a winter feeding ground for migratory wild fowl. At the end of 1929, however, about 90 per cent of the total water area was observed to be barren of seed plants. The relatively small quantity of plants, the most important of which were *Potamogeton pectinatus* L., *Potamogeton perfoliatus* L., *Vallisneria spiralis* L., *Naias flexilis* (Willd.) Rostk. & Schmidt, and *Ruppia maritima* L., remaining at that time was limited to clear, shallow waters in relatively isolated sections or along the shores of the larger bodies of water. The destruction of these seed plants was attributed to the chemical, physical, and biological conditions in the water, caused by the opening of the Albemarle and Chesapeake Canal, which permitted polluted sea water to flow from Chesapeake Bay and Norfolk Harbor into Back Bay and Currituck Sound with every north wind. The most unfavorable conditions for plant growth in the region were always found in or near the mouth of this canal.

Monthly analyses, made continuously for more than four years, showed the waters of Back Bay and Currituck Sound to be characterized by a variable salt content, varying from an average of 4.5 per cent sea water in April, 1928, to 21.5 per cent in January, 1929. During the entire period of the investigation, however, the average salt content of the main bodies of water was from 7 to 10 per cent sea water and that in the mouth of the Albemarle and Chesapeake Canal about 17 per cent. This suggested that the water in this canal was the principal source of salt entering Back Bay and Currituck Sound.

Another pronounced characteristic of the water of this region was its extreme turbidity. Measurements made throughout the seasons of 1929 and 1930 disclosed that very frequently all the radiant energy was absorbed by the first three feet of water. The average transmission of light to the bottom of Back Bay and Currituck Sound was found to be less than 2.0 per cent of the total solar energy present at the surface. The greatest turbidities were recorded in Currituck Sound near the mouth of the Albemarle and Chesapeake Canal.

Other marked characteristics of the water were a practically neutral reaction, a relatively high content of dissolved carbon dioxide, and a low content of dissolved oxygen. The carbon dioxide content was found to vary from 4 to 30 parts per million and the oxygen from 0.67 to 6.98 parts per

million. The higher content of carbon dioxide and the lower content of dissolved oxygen were recorded in the mouth of the Albemarle and Chesapeake Canal.

It was determined in the field experimentally that plants would not grow under the existing conditions in the barren areas. Furthermore, plants growing in waters contiguous to these areas were observed to be greatly damaged by colonial growths of the brackish-water hydroid, *Cordylophora lacustris* Allman, and by a disease induced by a fungus, previously identified as a strain of *Rhizoctonia solani* Kühn. Plants were found to grow well, however, in such waters when the entrance of suspended matter and injurious organisms from the larger bodies of water was prevented by the use of suitable bulkheads, which acted as filters.

Physiological studies were undertaken under controlled conditions in the laboratory in order to determine the influence of salinity, light, carbon dioxide supply, oxygen, mineral nutrients, and substratum on the growth of aquatic angiosperms, and to obtain more conclusive evidence on the causes of the destruction of the duck-food plants in Back Bay and Currituck Sound than that disclosed by the results of ecological studies.

Under favorable conditions of soil, light, temperature, and carbon dioxide supply, it was found that the growth of *Potamogeton pectinatus* and *Potamogeton perfoliatus* was stimulated by dilutions of sea water, the optimum concentration being 20 per cent sea water. Growth was retarded but not entirely inhibited by a concentration of 36 per cent sea water. The growth of *Naias flexilis* was increased only slightly in concentrations up to 4 per cent and that of *Vallisneria spiralis* and *Ceratophyllum demersum* retarded by very small amounts of sea water. These plants, however, survived in 20 per cent sea water, which was a higher content of salt than the average for the water of Currituck Sound and the maximum for Back Bay.

Potamogeton pectinatus, the dominant angiosperm in Back Bay and Currituck Sound, was found to require for growth and development the transmission to the bottom of culture vessels of more than 4 per cent of the total radiant energy. This quantity of light was about twice as much as average transmissions recorded at the bottoms of Back Bay and Currituck Sound.

Concentrations of dissolved carbon dioxide from 8 to 72 parts per million in cultures containing tap water or dilute nutrient solutions, with and without substrata, increased the growth of *Naias flexilis*, *Potamogeton perfoliatus*, and *Potamogeton foliosus*. The addition of oxygen to the culture solutions retarded rather than promoted the growth of these plants. The plants in the presence of light and furnished with a favorable carbon dioxide supply and sufficient mineral nutrients were found to produce in the process of photosynthesis more oxygen than was necessary to meet their own requirements for respiration. These results suggested that the con-

tents of carbon dioxide and oxygen probably are not important factors influencing the growth of angiosperms in the waters of Back Bay and Currituck Sound.

Sachs' nutrient solution diluted with tap water (1 to 30) stimulated the growth of *Naias flexilis*, *Potamogeton perfoliatus*, and *Potamogeton foliosus*. The plants were found to thrive in such a solution without a substratum, although not so well as with a soil substratum.

It was determined that tap water contained insufficient contents of mineral nutrients and dissolved carbon dioxide for an appreciable promotion of growth in three species of experimental plants. Of these two factors, the deficiency of mineral nutrients was considered the more important.

The results of experiments conducted for the purpose of determining the rooting response of *Naias flexilis*, *Potamogeton perfoliatus*, and *Potamogeton foliosus* cuttings suggested that the roots of aquatic plants are important as organs of absorption, and that their development is not stimulated by mere contact with a substratum but by the same factors which promote the growth of the intact plant.

From the results of the ecological and physiological studies reported in this paper it was concluded that unfavorable light conditions and the presence of certain injurious organisms are now the most important factors limiting the growth of angiosperms in the water of Back Bay and Currituck Sound. It was concluded also that the opening of the Albemarle and Chesapeake Canal produced in the main parts of these waters a change from a natural oligosaprobic condition to one approaching the polysaprobic in which aquatic angiosperms are found not to exist.

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EFFECT OF VARIOUS FACTORS ON THE pH OF PEATS

M. M. McCool

INTRODUCTION

The use of domestic peats for mineral soil improvement is increasing. Some users of them are interested only in the very acid ones and desire to maintain the pH of the media in which the plants grow within rather narrow ranges. Others desire only the less acid peats. Since the reaction or the pH values of peats may be affected by several factors it was considered to be advisable to study the changes induced by varying the ratio of peat to water, the effect of drying, leaching, the removal of fine material, and the addition of fertilizer salts and sulphur.

The peats employed in the investigation herein reported with few exceptions are described in a previous paper (10) but for reference a brief description is given: Peat Nos. 1, 2, and 3 are sandy and silty organic debris; No. 4, woody reed and sedge; Nos. 5, 6, 11, and 12, reed and sedge; No. 7, sedge-reed; No. 8, woody with sedge and reed; No. 9, reed and hypnum; No. 10, reed and sphagnum; No. 13, wood fibrous sedge; Nos. 14 and 17, sedimentary fibrous sedge; No. 15, sphagnum; No. 16, woody fibrous; Nos. 18 and 19, undetermined dark brown sticky; No. 20, sedimentary with diatoms; No. 21, unclassified sedimentary; No. 22, woody; No. 23, silty sedimentary.

REVIEW OF LITERATURE

Hitchcock (7) ascertained that greatly diluting a heavy suspension of sphagnum peat with water did not alter appreciably its pH value. The dilution of the peat extract, however, caused a marked change in pH value.

Feustel and Byers (5) found a proportion of about one part of peat to five parts of distilled water to give the best results. They determined the reaction of the peats in the fresh condition by means of the hydrogen electrode.

Johnson (8) surveyed the earlier literature on the subject of heat treatments of soils and in addition contributed much additional data. He found that dry heating of an air-dried muck and a peat soil to 25° C. markedly increased the concentration of the water extracts as determined by the freezing point method.

Bouyoucos and McCool (4) reported that autoclaving a Michigan peat high in water content for three hours at a pressure of 15 atmospheres resulted in a considerable increase in its freezing point lowering.

McCool (9) found great variations in the rate at which Michigan peats produced or released soluble material. The most active portion was the

first six inches of the deposits. In some the rate at which soluble materials formed was increased tremendously by increasing the temperature whereas with others this treatment was not so effective. He also calls attention to the possibilities in the use of certain peats in greenhouses and elsewhere.

It has long been known that sphagnum peats, when placed in contact with neutral salt solutions, become more acid in reaction than they do when distilled water is added to them. Skene (12) reviewed the early literature on the effect of soluble salts on the pH of sphagnum and repeated some of the previous studies and extended the researches somewhat farther. He found, upon the addition of five per cent salt solution to sphagnum, a strong acid reaction with methyl orange. The same indicator used with a distilled water extract of the sphagnum did not give these results. He also added 100 cc. solutions of copper chloride of 0.5, 0.1, 0.05, and 0.025 per cent respectively to about five grams of moist sphagnum and allowed them to stand overnight. Upon testing the original solutions and the extracts with ammonium hydroxide, he found that the color afforded by the stronger solutions in contact with the sphagnum to be much weaker and that by the two weakest ones to be almost, if not entirely, gone. Upon testing the extracts and the original solutions for chlorides with silver nitrate, the amount of the precipitate was the same before and after treatment. Much of the copper had been removed from solution by the sphagnum. The acid radicle was undiminished and the solution had become strongly acid in reaction. Skene then washed the sphagnum, which had been treated with salt solutions, with water saturated with carbon dioxide and found that 90 per cent of the base which had been removed from solution could be washed out. As the washing proceeded the acidity increased.

Arrhenius (2) included a sample of sphagnum peat in his investigations on the influence of neutral salts on the soil reaction. The concentrations employed ranged from 0.3 to 0.00003. He found the greatest increase in acidity to result from the addition of $FeCl_2$, next in order was $CaCl_2$, followed by $MgCl_2$. No changes were observed until a concentration of 0.003 was reached.

Aarnio (1) treated sphagnum peat (pH 4.72) with solutions of KCl and $CaCl_2$ respectively, washed it until free of chlorides, then found the former to change the pH value to 6.11 and the latter brought it to 5.14. These results are the opposite from those obtained upon treating mineral soils with these salts. However, he does not state whether a sample of the untreated peat was leached in a similar manner.

Gillespie and Wise (6) extracted humus from Superior clay soil type and found $BaCl_2$ to be more active than KCl or NaCl in releasing acidity. He varied the concentration of the salts used and employed the hydrogen electrode to determine the pH changes.

MATERIALS AND METHODS

In the studies on the effect of varying the ratio of peat to water on the pH of peats, with the exception of the partly decayed sphagnum, the samples were employed in the moist condition, or about as they were received from the deposits. A separate sample was taken for each amount of water and all stood 18 hours after the addition of water before the pH values were determined. The quinhydrone electrode was employed in making the determinations. Where the water content was not great enough to make a suspension, the sample for study was obtained by pressing the mass of peat against the side of the container by means of a spatula. The pH values of the moist, air-dried, and oven-dried peats were determined by employing the dry peat water ratio of 1 to 3. Ten grams of moist peat and 25 cc. of water were placed in test tubes and the tubes plugged with cotton. They were heated in the autoclave three hours under 15 lb. pressure and at a temperature of 255° C. Eighteen hours later the pH values of the heated and unheated samples were determined. Büchner funnels 12 cm. in diameter and 5 cm. deep were employed as containers in the studies on the effect of leaching on the pH of peats. The samples were placed on filter paper in the funnels and distilled water added. The water was slowly drawn through the mass by means of a vacuum pump. After the desired amount of water had passed through the peat, five-gram samples were removed, placed in glass beakers, 15 cc. of distilled water added, and the pH values determined 18 hours later. Samples of the last 50 cc. passing through were taken for pH readings.

In order to determine the effect of fine materials on the pH value the peats were dispersed in the Bouyoucos (3) dispersing machine for ten minutes, placed in 1000 cc. cylinders which were filled with distilled water, and allowed to stand 15 minutes. The supernatant liquid with the material in suspension was removed, the cylinder again filled with water, shaken, and the process repeated until the water was clear upon standing the allotted time. The residue was removed from the cylinder, collected on a Büchner funnel, and the excess water removed by suction. Ten-gram samples were weighed out, placed in beakers, and 50 cc. of distilled water and M/10 $MgCl_2$ respectively added. After 18 hours the pH values were determined. To serve as controls, samples of several of the peats were dispersed and leached as usual with the same amount of water required to remove the material which remained in suspension 15 minutes.

The effect of different amounts of sulphur on the acidity of peats was determined by mixing powdered sulphur with two gallons of peat. The water content of peat No. 15 was maintained at 75.5 per cent and the others at 70 per cent. They were incubated at room temperature. One hundred grams of peat No. 15 with 200 cc. of distilled water and 50 grams of the other peats with 100 cc. of distilled water were used in making the

TABLE I

pH OF PEATS AT DIFFERENT WATER CONTENTS

Peat No.	Weight, g.	Water content, %	Water added to moist sample, cc.	pH	pH change
3	25	80	0	4.78	0
			25	4.68	−0.10
			56	4.88	+0.10
			75	4.97	+0.19
			100	5.04	+0.26
			150	5.19	+0.41
			250	5.31	+0.53
4	50	50	15	5.97	0
			25	6.03	+0.06
			50	6.07	+0.10
			100	6.07	+0.10
			200	6.27	+0.30
			300	6.41	+0.44
			500	6.44	+0.47
5	50	72	0	5.90	0
			15	5.88	−0.02
			25	5.93	+0.03
			50	5.93	+0.03
			100	5.97	+0.07
			200	6.14	+0.24
			300	6.14	+0.24
			500	6.09	+0.19
6	50	70	0	5.65	0
			10	5.81	+0.16
			25	5.70	+0.05
			100	5.65	0
			200	6.12	+0.47
			300	6.25	+0.60
			400	6.25	+0.60
			500	6.25	+0.60
7	25	82	12.5	5.70	0
			25	5.71	+0.01
			50	5.76	+0.06
			100	6.10	+0.40
			200	6.05	+0.35
			500	6.05	+0.35
8	25	88	0	5.88	0
			20	5.98	+0.10
			50	6.05	+0.17
			500	6.46	+0.58
9	25	86	0	5.93	0
			20	6.19	+0.26
			40	6.27	+0.34
			500	6.53	+0.60

TABLE I (*continued*)
pH OF PEATS AT DIFFERENT WATER CONTENTS

Peat No.	Weight, g.	Water content, %	Water added to moist sample, cc.	pH	pH change
10	25	80	0	3.29	0
			5	3.23	−0.06
			10	3.26	−0.03
			25	3.23	−0.06
			125	3.48	+0.19
			250	3.67	+0.48
			500	3.85	+0.56
11	50	80	0	5.39	0
			5	5.68	+0.29
			15	5.83	+0.44
			25	5.80	+0.41
			200	6.22	+0.83
			300	6.25	+0.86
			500	6.39	+1.00
12	50	62	15	5.73	0
			20	5.66	−0.07
			30	5.68	−0.05
			100	5.85	+0.12
			150	6.27	+0.54
			300	6.37	+0.64
			500	6.37	+0.64
13	25	85	0	4.85	0
			10	4.92	+0.07
			15	5.07	+0.22
			25	4.99	+0.14
			250	5.18	+0.33
			500	5.46	+0.61
15	50	65	0	3.48	0
			10	3.38	−0.10
			30	3.48	0
			50	3.48	0
			100	3.61	+0.13
			250	4.17	+0.69
			500	4.14	+0.66
18	25	74	50	3.29	0
			100	3.43	+0.14
			200	3.60	+0.31
			500	3.85	+0.56
22	25	80	25	5.21	0
			50	5.21	0
			100	5.58	+0.37
			200	5.97	+0.76
			500	6.22	+1.01
23	25	59	50	5.07	0
			100	5.54	+0.47
			200	5.88	+0.81
			500	6.10	+1.03

pH determinations. After the addition of the water the mass was stirred several times, let stand 24 hours, again stirred, and the readings taken.

RESULTS

pH of peats at different water contents. Although the hydrogen ion concentrations of numerous peats have been reported, the effect of varying the peat to water ratio on the pH values of widely different peats remains to be done.

According to the data in Table I the pH of peat Nos. 3, 4, 6, 8, 9, 10, 12, 13, 15, and 18 showed slight but similar changes upon dilution with distilled water. Peat Nos. 5 and 7 changed less in this respect and the pH values of peat No. 11, the woody peat No. 22, and the silty sedimentary peat No. 23, were increased about pH 1.0 by the highest dilution. According to the results a definite water to peat ratio should be used if rather close comparisons are to be made and if studies of the effects of different treatments are to be conducted.

These results raise the question as to the pH of the soil mass with which the roots come into contact. It may be different from that of the suspensions employed in the usual procedure and it is doubtful if the true value has been determined.

Effect of drying on the pH of peats. It is not always practical to determine the reaction of peats before they become dry. The reaction of some may change upon the removal of moisture from them and thus result in erroneous conclusions with respect to their value for different tests. Experiments were conducted to determine to what extent air-drying and oven-drying affected pH values of different peats. According to the data presented in Table II, the changes induced in the reaction by drying although not great varied considerably. If, as assumed, differences of more than pH 0.2 are significant, the hydrogen ion concentration of the air-dried peats, Nos. 4, 7, 8, 11, and 12, was less after 24 hours than it was 10 minutes after distilled water was added to them. Peat Nos. 3, 13, and 22 became more acid upon standing. The pH values of the oven-dried peats, Nos. 1, 2, 4, 7, 13, and 18, were significantly greater 10 minutes after the addition of distilled water than they were 24 hours later. With the exception of peat Nos. 1, 2, and 13 the reaction of the moist peats did not change upon standing.

A comparison of the results obtained from the air-dried with the moist peats at the end of 10 minutes shows the hydrogen ion concentration of moist peat Nos. 3 and 22 to be greater and that of No. 21 to be less than that of the air-dried samples. Twenty-four hours later the pH values of the moist peats, Nos. 1, 4, 5, 7, 8, and 11, were lower than those of the corresponding air-dried samples, and those of No. 21 greater.

The pH values of the oven-dried and the moist peats, Nos. 1, 2, 3, 4, 5,

TABLE II

pH OF AIR-DRIED, OVEN-DRIED, AND MOIST PEATS

Peat No.	Air-Dried		Oven-dried		Moist	
	Time after addition of water		Time after addition of water		Time after addition of water	
	10 min.	24 hrs.	10 min.	24 hrs.	10 min.	24 hrs.
1	5.98	6.03	5.93	5.54	5.92	5.5[illegible]
2	5.66	5.58	5.75	5.44	5.73	5.41
3	4.80	4.51	4.65	4.56	4.56	4.56
4	5.44	5.70	5.61	5.36	5.61	5.44
5	5.70	5.78	5.61	5.53	5.65	5.58
6	5.90	5.85	5.54	5.48	5.98	5.83
7	5.19	5.43	5.10	4.82	5.19	5.07
8	6.24	6.46	5.95	5.87	6.41	6.24
9	6.00	6.01	5.54	5.43	6.00	5.96
10	3.53	3.50	3.72	3.48	3.48	3.39
11	5.87	6.37	5.95	5.80	6.03	5.84
12	5.63	5.78	5.78	5.78	5.81	5.65
13	5.53	5.24	5.19	4.97	5.53	5.14
14	5.63	5.49	5.28	5.34	—	—
18	3.68	3.60	3.72	3.51	3.58	3.58
21	3.43	3.34	3.34	3.24	4.17	4.02
22	5.71	5.04	4.90	4.78	5.34	5.19

10, 11, 12, and 18, after 24 hours agreed closely and those for the oven-dried peats, Nos. 6, 7, 8, 9, 21, and 22, were lower than were those of the corresponding moist peats.

Effect of drying on the soluble salt content of peats. The freezing point lowering of the dried and moist peats was determined in order to ascertain if there was any relationship between the changes in the soluble salt content and the pH values of the peats. The data obtained from these studies are presented in Table III.

TABLE III

EFFECT OF DRYING ON THE FREEZING POINT LOWERING OF PEATS IN °C.

Peat No.	Air-dried			Oven-dried			Moist		
	Time after addition of water			Time after addition of water			Time after addition of water		
	10 min.	24 hrs.	48 hrs.	10 min.	24 hrs.	48 hrs.	10 min.	24 hrs.	48 hrs.
3	0.041	0.033	0.040	—	0.010	0.033	0.029	0.025	0.021
4	0.057	0.058	0.061	0.110	0.135	0.136	0.053	0.060	0.037
5	0.044	0.040	0.037	0.070	0.094	0.088	0.029	0.038	0.014
6	0.040	0.021	0.020	0.045	0.055	0.059	0.020	0.028	0.011
7	0.047	0.048	0.050	0.075	0.123	0.128	0.050	0.069	0.051
8	0.050	0.031	0.036	0.060	0.070	—	0.028	0.024	0.029
9	0.036	0.021	0.020	0.020	0.028	0.040	0.025	0.023	0.016
10	0.037	0.018	0.026	0.030	0.048	0.053	0.019	0.020	0.075
11	0.127	0.120	0.129	0.216	0.249	0.248	0.058	0.069	0.036
12	0.042	0.032	0.037	0.039	0.060	0.061	0.027	0.025	0.030
13	0.011	0.010	0.013	0.020	0.036	0.041	0.017	0.011	0.021

A comparison of the freezing point lowerings of the air-dried and moist peats shows slightly lower values for 8 of the 13 moist peats after 10 minutes, 3 of the 13 after 24 hours, and 5 of the 13 after 48 hours. Air-drying peat No. 11 increased its soluble salt content greatly. Eight of the 12 oven-dried peats contained more soluble salts after 10 minutes than did the moist peats. Twenty-four hours later 9 of the 13 and after 48 hours 8 of the 12 contained more than did the moist peats. Differences in freezing point lowerings greater than 0.01° C. were considered significant in making the comparisons. The effect of drying peats on their value for mineral soil improvement remains to be studied. It may be that their value would be decreased owing to changes induced in their physical properties and the loss of soluble constituents by leaching.

TABLE IV
EFFECT OF LEACHING ON THE pH OF VERY ACID PEATS

Water used, cc.	Peat No. 10		Peat No. 15	
	Solid	Leachings	Solid	Leachings
0	3.12	—	3.38	—
100	—	3.16	—	3.38
100	—	3.33	—	4.17
300	—	3.21	—	3.60
1st 1000	3.19	3.80	3.61	4.16
2nd "	3.31	4.68	3.61	5.04
3rd "	3.50	4.75	3.65	4.33
4th "	3.50	5.61	3.67	4.85
5th "	3.41	5.53	—	4.34
6th "	3.63	5.53	3.75	5.00
7th "	3.65	5.12	3.75	5.02
8th "	3.75	5.12	3.87	5.05
9th "	3.99	4.60	3.87	4.56
10th "	3.89	4.60	3.83	4.38
15th "	4.93	5.27	4.63	5.12
20th "	5.27	5.08	4.80	5.56
25th "	5.21	6.24	5.49	6.12
25th "	5.22*	—	5.35*	—

* After 18 days.

Effect of leaching on the pH of peats. Does the pH of peats change upon leaching? This is an important question not only from the scientific standpoint but also from the applied aspect. Several peats were slowly leached with distilled water and the changes in their pH values and those of the leachings determined. According to the data presented in Table IV, the very acid peats, Nos. 10 and 15, changed slowly upon leaching. The pH of the former before leaching was 3.12, and after 25 liters of leaching it was 5.21. The values for the latter were 3.38 and 5.35 respectively. The leachings also became less acid as the leaching continued, but more rapidly than the solid portions. The somewhat less acid peat, No. 3, reacted in a similar manner. The peats whose pH values were 6 or greater changed but little on leaching (Table V).

TABLE V

Effect of Leaching on the pH of Less Acid Peats

Water used, liters	Peat Nos. 3 Solid	3 Leachings	6 Solid	6 Leachings	9 Solid	9 Leachings	11 Solid	11 Leachings	12 Solid	12 Leachings	13 Solid	13 Leachings
1	4.77	6.14	6.32	6.12	6.31	6.05	6.44	6.59	6.12	6.63	7.59	6.90
3	4.82	5.81	6.49	6.29	6.41	6.20	6.66	6.70	6.25	6.63	7.59	6.73
5	4.99	5.92	6.58	6.20	6.51	6.29	6.53	6.05	6.32	6.41	7.84	6.39
10	5.53	6.42	6.58	6.20	6.54	6.12	6.50	6.80	6.46	6.73	7.79	7.08
15	5.36	6.12	6.17	6.63	6.25	6.64	6.61	6.47	6.46	6.27	7.64	7.17
*	5.36	—	6.54	—	6.37	—	6.81	—	6.57	—	7.32	—

* After 18 days.

The leached samples were kept moist 18 days and their hydrogen ion concentrations again determined. Peat Nos. 3 and 10 did not change but No. 15 became slightly more acid. The less acid peats, Nos. 6, 9, and 11, became slightly more acid upon standing, and No. 13 changed to a greater extent. The very acid peats when leached with 5 and 10 liters of water respectively returned to the original pH upon standing in the moist condition.

Effect of grinding dried peats on their pH values. In order to determine if the changes in structures resulting from drying were responsible for the above results, several of the dried peats were finely ground and the pH values determined after 10 minutes and 24 hours respectively. This treatment had very little effect on the hydrogen ion concentration of the dried peats.

Effect of heat on the pH of peats. Peat and mineral soil mixtures are subjected to steam and to heat under pressure to rid them of insects, fungi and bacteria. In order to determine whether such treatments alter the hydrogen ion concentration, moist samples of peats were subjected to heat under pressure. The pH of peat No. 9 was decreased 0.38 and that of peat No. 21 was increased 0.57. The reaction of the remainder of peats was not altered by this treatment.

As judged by the results obtained from the drying, grinding, heating, and leaching studies, the pH values of peats are rather stable.

Effect of the removal of fine material on the pH of peats. Several peats were dispersed and the material remaining in suspension for 15 minutes was removed.

The pH values of the residue, the dispersed and leached, and the dispersed and unleached samples were determined in order to ascertain the effect of the fine material on their acidity. The removal of the suspended matter in this manner raised the pH values of the strongly acid peats greatly, or nearly to the neutral point (Table VI). The less acid peats responded much

TABLE VI

EFFECT OF THE REMOVAL OF FINE MATERIAL ON THE pH OF PEATS

Peat No.	Residue*		Dispersed and leached		Dispersed and unleached	
	H_2O	$MgCl_2$ M/10	H_2O	$MgCl_2$ M/10	H_2O	$MgCl_2$ M/10
2	5.88	5.12	6.07	5.00	5.54	4.73
4	6.98	5.53	7.04	6.54	6.22	5.63
5	6.85	5.80	—	—	6.39	5.54
6	6.75	5.85	6.07	—	6.39	5.75
8	6.63	5.51	7.02	6.76	6.47	5.63
9	6.64	5.54	6.48	6.22	6.22	5.43
10	6.64	5.14	3.80	3.46	3.46	2.85
11	6.70	5.43	7.81	7.03	6.71	5.87
12	6.64	5.54	7.15	6.42	6.42	5.44
15	6.74	5.24	3.80	3.15	3.70	2.55
16	5.88	—	5.73	4.70	—	—

* Dispersed 10 minutes with Bouyoucos' electrical apparatus.

less strikingly to these treatments. The pH of the residues of some was slightly less, others slightly greater, and that of still others was the same as the dispersed and leached samples. The addition of M/10 magnesium chloride to the differently treated samples resulted in a greater increase in the acidity of the residues than that of the dispersed and leached or of the dispered and unleached samples.

TABLE VII

EFFECT OF N/10 SALT SOLUTIONS ON THE pH OF PEATS

Salt	pH of salt solution	Peat Nos.						
		2	9	10	13	15	21	22
$Ca(NO_3)_2$	4.85	4.73	5.12	2.80	4.42	2.65	5.07	4.70
$NaNO_3$	6.37	5.41	5.80	3.33	5.15	3.36	—	—
NH_4NO_3	6.27	5.37	5.73	3.23	5.04	3.23	—	—
NH_4Cl	6.37	5.59	5.70	3.24	5.02	3.28	—	—
$(NH_4)_2SO_4$	6.20	5.42	5.81	3.24	5.19	3.33	5.54	5.12
KCl	6.56	5.36	5.78	3.31	5.02	3.33	—	—
KNO_3	6.59	5.40	5.78	3.34	5.05	—	—	—
H_2O	—	6.00	6.03	3.50	5.39	3.70	6.10	5.54

Effect of fertilizer salts on the pH of peats. Certain peats were treated with several nutrient salts, superphosphate, and a commercially mixed fertilizer, and their effect on the hydrogen ion concentration determined.

In the first series 50 cc. of N/10 salt solutions were added to 50 grams

of several peats (moisture content of which was 75 per cent), stirred, let stand 24 hours, again stirred, and the pH values determined. The results of these determinations are given in Table VII. The changes induced by calcium nitrate were greater than were those brought about by the other salts.

More dilute solutions or N/50 and N/250 were employed in another series. Although the data are not given the N/50 calcium nitrate increased the acidity somewhat and the changes induced by the same concentration of the other salts were of smaller magnitude. The N/250 solutions did not alter the reading sufficiently to be considered positive in their effects. From the practical standpoint it is evident that extremely large applications of these salts are necessary in order to lower the pH values of these peats.

TABLE VIII
Effect of Fertilizers on the pH of Peats

Peat No.	Ratio of superphosphate to peat			Ratio of 4–8–7 fertilizer to peat			Control
	1:100	1:250	1:500	1:50	1:100	1:500	
14	6.90	7.40	7.49	7.32	7.32	7.56	7.57
15	3.38	3.43	3.50	3.41	3.41	3.53	3.68
18	3.17	3.28	3.43	3.28	3.26	3.39	3.58
21	5.10	5.39	5.51	5.58	5.51	5.56	5.76

Superphosphate (16 per cent P_2O_5) and a 4–8–7 commercial fertilizer were finely powdered and added to moist peats in different proportions. One part of the mixture of peats and fertilizers received two parts of water. Seventy-two hours later the pH values were determined. As the data in Table VIII show, the presence of one part of superphosphate in 100 parts of peat affected the pH of the very acid peats about one-half as much as it did the others. The ratio of one part of phosphate to 250 of peat increased the acidity to a less extent and the changes induced by the addition of one part of phosphate to 500 of peat were very slight. The addition of one part of the mixed fertilizer to either 50 or 100 parts of the peats increased their acidity to a slight extent. The presence of one part of it in 500 parts of the peats was less effective in lowering the pH values. Thus very heavy applications of these materials are necessary in order to increase markedly the acidity of the peats.

Monobasic ammonium, sodium, and potassium phosphate were added to several peats and the changes in the hydrogen ion concentration determined. Fifty cc. of the solutions were added to 25 grams of the moist peats and the pH values determined 48 hours later. In the first series of experiments ammonium phosphate was used in different concentrations. According to the data in Table IX the M and M/10 solutions increased the acidity of the less acid peats. The results obtained from the strongly acid peats,

Nos. 10, 15, and 19, were interesting. The pH values of these were not altered by the M solution but they were lowered by the M/10 solution.

Another series of experiments was conducted in which sodium and potassium phosphate were added to the acid peats. The results were similar to those obtained from ammonium phosphate.

The pH of the M ammonium phosphate was 3.67, of the M/10 4.22; the M sodium phosphate was 3.81, M/10 4.39; and the M potassium phosphate 4.04, and M/10 4.49.

TABLE IX

EFFECT OF MONOBASIC AMMONIUM PHOSPHATE ON THE pH OF PEATS

Peat No.	Concentration		
	M	M/10	Control
3	3.85	4.60	6.08
9	3.47	5.14	6.03
10	3.73	3.48	3.72
11	4.38	5.14	6.75
14	3.43	3.26	3.43
15	3.63	3.36	3.63
16	4.12	4.85	5.31
19	3.43	3.17	3.39

The amounts of ammonia removed from solution, the calcium and magnesium displaced, and the fixation of phosphate when peat No. 10 was treated with M ammonium phosphate were determined. The peat removed 0.588 of a gram of NH_4 from the ammonium phosphate, and released 0.012 of a gram of calcium and 0.008 of a gram of magnesium when treated with this salt solution. The fixation of the phosphorus by the peat amounted to 0.374 of a gram. McGeorge (11) found very acid peat to contain relatively small amounts of replaceable calcium, magnesium, sodium, and potassium but large amounts of replaceable hydrogen. It seems that the ionic changes taking place are complicated by the existence of different forms of phosphate ions. Any attempt to balance the various ions involves some assumptions regarding the relative concentrations of the several phosphate ions.

Effect of sulphur on the pH of peats. Growers of certain plants desire rather strongly acid peats for their culture. Sphagnum peat has been utilized for this purpose by some, while others have become interested in methods for increasing the acidity of the domestic peats.

In order to determine the extent and rate of change in the reaction, powdered sulphur was added to several peats and the changes in pH after different incubation periods were determined. In the first series of experiments the sulphur was added to peat Nos. 14 and 15, the mixture place in two-gallon glazed jars and allowed to stand at room temperature. The water content of the former was 70 per cent and of the latter 75 per cent.

TABLE X

EFFECT OF SULPHUR ON THE pH OF PEATS

Culture No.	Peat No.	Sulphur to peat ratio	Duration of contact in days											Decrease in pH during 140 days
			0	10	20	30	44	56	69	82	94	112	140	
1	15	1:1000	3.45	3.33	3.24	2.60	2.45	2.50	2.40	2.58	2.46	2.48	2.40	1.05
2	15	1:500	3.48	2.79	2.51	1.94	1.80	1.97	1.99	1.62	1.62	1.63	1.73	1.75
3	15	1:250	3.41	2.63	2.13	1.82	1.79	1.92	1.89	1.43	1.62	1.53	1.55	1.86
4	14	1:1000	6.98	7.07	6.46	5.88	5.53	4.58	5.68	4.17	5.10	4.55	4.88	2.10
5	14	1:500	6.96	6.86	6.15	5.04	4.90	4.31	4.09	4.66	4.33	3.99	4.22	2.74
6	14	1:250	7.00	6.73	5.68	4.88	4.63	3.83	3.58	3.46	3.02	3.06	2.90	4.10

According to the data in Table X rather marked changes took place in peat No. 15. The increase in its acidity was small after the close of the 30-day incubation period. The changes in the hydrogen ion concentration of peat No. 14 not only were greater but also continued over longer periods than in the case of peat No. 15.

Seven additional peats were employed in the second series of experiments. In this series one part of sulphur was mixed with 250 parts by weight of peat. The moisture content of the peats was 75 per cent. The results obtained from this series are given in Table XI. Peat No. 7 was the most active in the oxidation of sulphur followed in order by Nos. 5, 6, 12, 8, 9, and 3. Judging from these results the acidity of peats or peat and mineral soil mixtures may be increased satisfactorily by the addition of sulphur.

One hundred grams of sulphur-treated peats, Nos. 15 and 14, were leached 15 days with 7000 cc. of distilled water in order to determine the rate at which the pH values could be changed. The pH of the former before leaching was 2.02 and the latter was 1.75. At the close of the period the pH of the former was 4.09 and the latter was 3.04. Thus the acidity resulting from the addition of sulphur to peats appears to be difficult to change by leaching.

SUMMARY

Several factors which may alter the hydrogen ion concentration of peats have been studied.

The pH values of the reed and sedge peat No. 5 and the sedge-reed peat No. 7 changed to a small extent upon widening the peat to water ratio. The values for reed and sedge peat No. 11, the sedimentary peat No. 21, and the silty sedimentary peat No. 23 became about 1.0 greater upon high dilution.

The changes in the reaction of the remainder of the peats induced by dilution were slight.

Thirteen peats were air- and oven-dried and their pH values, as well as those of the moist peats, were determined after 10 minutes and 24 hours. The change induced in some was slight, but in others it was greater. Those which were affected to the greatest extent were the oven-dried reed and sedge peat, woody with sedge and reed peat, reed and hypnum peat, the air- and oven-dried sedimentary peat, and the woody peat.

Air-drying increased the soluble salt content of several of the peats and oven-drying resulted in a greater concentration of the solution of each of them. There was no correlation between the changes induced in the pH values and the soluble salt content of the peats by drying.

TABLE XI

EFFECT OF SULPHUR ON THE pH OF PEATS

Peat No.	Duration of contact in days								Decrease in pH during 120 days
	0	13	25	47	57	74	90	120	
3	4.63	4.48	4.19	3.75	3.92	2.87	2.63	2.64	1.99
5	5.71	4.93	4.66	4.48	4.38	4.26	4.09	4.09	1.62
6	5.80	5.09	4.99	4.68	4.55	4.44	4.14	4.12	1.68
7	5.95	4.65	4.27	3.97	3.99	3.95	3.92	3.96	1.99
8	5.51	4.73	5.02	4.87	4.82	4.78	4.51	4.54	0.97
9	5.80	5.21	5.12	4.77	4.71	4.56	3.92	3.95	1.85
12	5.71	5.02	4.92	4.29	4.20	4.10	4.05	4.08	1.63

The hydrogen ion concentration of the very acid peats became markedly less upon leaching with distilled water. The less acid peats changed much less in this respect.

It was evident that the fine material present in the very acid peats was largely responsible for their low pH values since upon its removal they became much less acid.

The addition of N/10 $Ca(NO_3)_2$ increased the hydrogen ion concentration of the peats. Less striking changes were obtained with the addition of N/10 solution of KCl, KNO_3, NH_4Cl, NH_4NO_3, $(NH_4)_2SO_4$, and $NaNO_3$. Monobasic sodium, potassium, and ammonium phosphate reacted in a similar manner with the less acid peats. The M solutions did not alter the pH values of the stronger acid peats and M/10 affected them but slightly.

Large additions of commercial superphosphate (16 per cent P_2O_5) and a 4–8–7 commercial fertilizer were required to alter the pH values of the peats.

The addition of powdered sulphur to the moist peats lowered their pH values. The rate of change was more rapid in some than it was in others. The acid resulting from the addition of the sulphur was difficult to remove by leaching with water.

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EFFECT OF ETHYLENE CHLORHYDRIN VAPORS UPON DORMANT LILAC TISSUES[1]

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Since the vapors of ethylene chlorhydrin had been found (5) to be effective in breaking the dormancy of lilac, *Syringa vulgaris* L., experiments were undertaken for the purpose of measuring some of the changes taking place in the tissue between the time of treatment and visible swelling of buds.

At intervals of two to four days after the treatment branches were removed from the potted plants, and tests were made upon the bud and bark tissue for catalase, amylase, and invertase activity, and analyses were made for moisture, sugars, starch, polysaccharides, and nitrogen. In addition measurements were made of the respiration of twigs either while they remained upon the plant or after removal from it.

The most pronounced differences induced by the treatment were increases in respiration, catalase, and soluble nitrogen, and decreases in sugars. Increases in moisture were marked in buds but not in the bark. These changes were observable within two or three days, and, in the case of the respiration, as early as the first day, after the end of the treatment.

METHODS

Treatments. The lilac plants, variety Charles X, were about three feet high and had been planted in 12-inch pots which were sunk into the ground so that the top of the pot was slightly above the surface of the soil. Good growth of the plants was obtained during the summer. In October the potted plants were brought in from the field and were allowed to remain outdoors until the treatments were applied. Two sheet-iron chambers, one with a capacity of 4500 liters, and the other of 4200 liters, were used for applying the chemical. The ethylene chlorhydrin was the commercial material, and contained approximately 40 per cent by weight of ethylene chlorhydrin ($ClCH_2CH_2OH$). The amounts applied were as follows: Nov. 10 and Nov. 16 treatments, 450 cc. in the 4500-liter chamber; Dec. 2 treatment, 375 cc., and Dec. 9 treatment, 350 cc., both in the 4200-liter chamber. The chemical was poured on cheesecloth of the proper amount to avoid dripping, and, after the cheesecloth was suspended in the room, an electric fan (with switch external to the room) was allowed to run for six hours, after which time the air in the room was not stirred. The total time of exposure to vapors was 22 hours.

Sampling. After treatment the pots of treated plants, usually eight, and an equal number of pots of untreated plants were placed in the green-

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house. At the intervals shown in the tables, branches were removed for the enzyme tests and chemical analyses. The lilac plants had been trained to produce three to six branches which spread out from near the surface of the soil. At the first sampling period one to three branches were taken from each plant, the other branches being left for subsequent samples. Thus, the tissue for each sample came from several plants, and tissue was furnished by these plants for successive samples. The buds, both terminal and lateral, were removed first, and the remaining portion was separated by pruning shears into portions representing the current year's growth and tissue older than that. These stems, representing tissue of different ages, were scraped with a knife, and the portions external to the woody cylinder were collected. Thus, three types of tissue were obtained: bud, bark of current season's growth (hereafter called twig-bark), and bark of older stems (hereafter called stem-bark). The woody portion was rejected, previous experiments having indicated that not only was it low in enzyme activity, but that the differences brought about by chemical treatment of the plants had less effect upon the wood than upon the buds and bark.

Enzyme tests. The tissue was passed first through a food grinder, once for bark and twice for bud tissue, and portions were weighed out for the enzyme tests. For catalase one gram of bud tissue or two grams of bark tissue were placed in a mortar and ground with twice the weight of calcium carbonate and twice the weight of sharp sand. After suitable disintegration the tissue was transferred to a volumetric flask with water and was made up to volume, to 200 cc. for the bud tissue and to 100 cc. for the bark tissue. After thorough mixing of this material, aliquots were removed with a pipette which had a coarse opening, 5 cc. being used for bud catalase and 10 cc. for bark catalase. The apparatus for the catalase determination was similar to that described by Davis (2), and the amount of oxygen delivered at the end of 0.5, 1.0, and 2.0 minutes was recorded. The hydrogen peroxide was neutralized by adding an excess of $CaCO_3$ and filtering. The procedure for preparing the tissue for the amlyase and invertase measurements was the same as that for catalase except that 8 grams of tissue were taken for the grinding and no $CaCO_3$ was added. The bud tissue was made up to 200 cc. and the aliquots taken were 10 cc. for the amylase and 50 cc. for the invertase measurements. The bark tissues were made up to 300 cc. from which 10 cc. were pipetted out for amylase and 50 cc. for invertase. To each lot were added 25 cc. of a buffer solution consisting of equal parts of one-third molar KH_2PO_4 and Na_2HPO_4. To the amylase lots were added 25 cc. of a 4 per cent solution of soluble starch, and to the invertase lots 25 cc. of an 8 per cent solution of sucrose. Water was added to make a final volume of 75 cc. for the bud tissue, and 100 cc. for the bark tissue. In addition to these lots containing added starch or sucrose there were blank lots similar in every way except that water instead of starch or

sucrose was added. The increase in reducing sugar in the lots containing tissue plus starch or sucrose over those containing tissue plus water was regarded as a measure of the amylase or invertase activity. The mixtures of tissue suspension, buffer, and water or enzyme substrate were placed in large test tubes, toluene was added, and, after rubber stoppers were firmly inserted and tied in with cord, the tubes were rotated slowly, end over end, on a turning bar inside an oven maintained at a temperature of 30° C. After 44 hours, the sample was added to alcohol to make the mixture 70 per cent alcohol by volume, and, after filtration, an aliquot was taken for the sugar determination. The alcohol was removed by evaporation on a steam bath, the aqueous solution was made up to volume, and the sugar was determined by the Munson and Walker method (1, p. 78), the cuprous oxide being titrated with a solution of potassium permanganate. The difference between the titration values obtained from the two lots, one in the presence and the other in the absence of starch or sucrose, was recorded as the amylase or invertase value for the amount of tissue in the sample.

Chemical analyses. The bud, twig-bark, and stem-bark tissue which had been passed through the food grinder but which was not used for the enzyme tests was used for the chemical analyses. After being weighed, the tissue was dropped into boiling 95 per cent alcohol which was then removed by evaporation on a steam bath. The residue was dried for 16 hours in an oven at 98° C., was weighed in order to obtain the dry weight and moisture values, was powdered in a mortar and was sifted through a 60-mesh sieve. Portions of this powder were weighed for analysis. The dry powder was extracted seven times successively with 70 per cent alcohol (by volume) and was centrifuged after each extraction. The residue which was insoluble in 70 per cent alcohol was used for the determination of starch, polysaccharides, and insoluble nitrogen. The soluble portion was used for the determination of soluble nitrogen and sugars. The Gunning method (1, p. 7) was used for the insoluble nitrogen, and for the soluble nitrogen the method of Pucher, Leavenworth, and Vickery (10) which includes nitrate nitrogen, was followed until that stage of the procedure at which the nitrates had been reduced by iron, after which the Gunning method was followed. The sugar determinations were made by the Munson and Walker method (1, p. 78) and the cuprous oxide was titrated with a potassium permanganate solution which had been standardized with a sugar solution of known concentration. Two methods of inverting sucrose were used, by acid hydrolysis in the cold (1, p. 75), and by the use of yeast invertase. The polysaccharides were estimated by the acid hydrolysis method (1, p. 95) and represent the acid-hydrolyzable substances insoluble in 70 per cent alcohol calculated as dextrose. The polysaccharide values include starch but a separate determination of starch was made by the

Walton and Coe method (12) which eliminates interfering polysaccharides. This method was modified by using saliva instead of malt diastase.

Respiration. For the measurement of the carbon dioxide formed by respiration, the twigs were cut from the plants and were put into 500 cc. Erlenmeyer flasks with a little water in the bottom to keep the twigs from drying out. The flasks were placed in a thermostatically-controlled water bath and a current of CO_2-free air was passed through. The CO_2 given off was absorbed in Van Slyke-Cullen (11) tubes containing saturated barium hydroxide. At the end of the experiment the barium carbonate was filtered off, thoroughly washed, dissolved in an excess of 0.1 N HCl, boiled, and

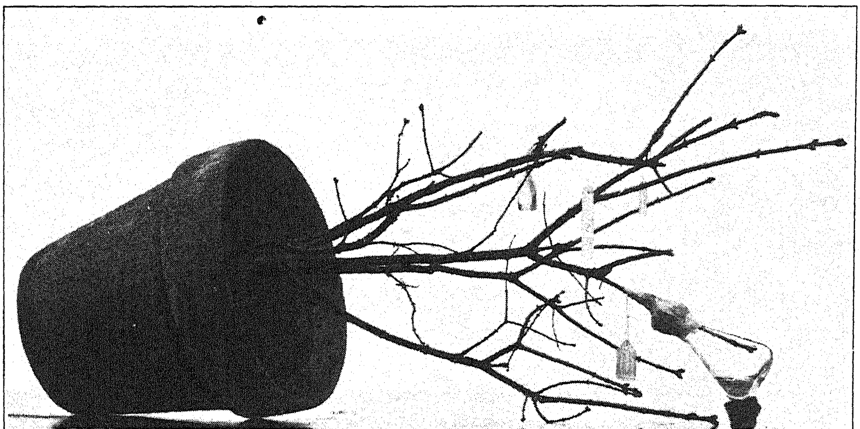

FIGURE 1. Method of measuring the respiration of a twig. Flask is sealed with modelling clay covered with paraffin. Carbon dioxide is absorbed by barium hydroxide solution in bottom of flask.

the excess of HCl was titrated with 0.1 N NaOH, using methyl red as an indicator. For the respiration measurements with attached twigs, the pots containing the plants were placed in a horizontal position and the twig was inserted into a 150 cc. Erlenmeyer flask to a length of about 10 cm. as is illustrated in Figure 1. The flasks were sealed by means of modelling clay which was then covered with paraffin. The flask contained a saturated solution of barium hydroxide to absorb the CO_2 given off. The amounts of CO_2 given off were not large enough to form a thick scum on the barium hydroxide and thus interfere with the absorption. As a precautionary measure, however, the flasks were gently shaken several times during the 24 hours in order to present fresh surfaces of the barium hydroxide for the absorption of CO_2. At the end of the experimental period, usually one day but sometimes two days, the flask was removed, the barium carbonate was filtered off, and the estimation of the CO_2 produced by the respira-

tion was carried out as described above. Another flask was attached in the same manner and the measurement of the respiration of the same twig for another period was made. Thus, the respiratory rates for individual twigs were obtained on successive days until about the fifth or sixth day after treatment.

RESULTS

EFFECT OF TREATMENT ON ENZYME ACTIVITY

The effects of the ethylene chlorhydrin treatment upon the enzyme activity of bud, twig-bark, and stem-bark tissue are shown in Tables I, II, and III. Examining first the results with bud tissue in Table I it is

TABLE I

EFFECT OF ETHYLENE CHLORHYDRIN TREATMENT UPON ENZYME ACTIVITIES OF BUD TISSUE

Date treated 1931	Days after treatment	Catalase cc. O_2 per 0.01 g.		Amylase cc. $KMnO_4$ per 0.04 g.		Invertase cc. $KMnO^4$ per 0.2 g.	
		Tr.*	Ck.	Tr.*	Ck.	Tr.*	Ck.
Nov. 10	3	7.1	4.9	11.1	11.6	15.1	5.9
	5	7.6	4.3	15.7	12.9	17.1	11.0
Nov. 16	2	4.3	3.6	5.2	8.8	12.1	10.8
	4	7.2	4.3	8.2	11.4	14.3	10.0
	6	7.0	3.4	9.5	7.2	13.9	10.5
Dec. 2	2	3.4	3.1	13.8	14.6	15.5	10.7
	4	6.5	2.6	18.9	17.5	12.8	10.7
	6	7.3	2.9	18.7	14.7	17.2	11.2
Dec. 9	4	4.9	3.4	18.4	20.6	13.3	10.7
	6	5.6	3.4	27.2	23.3	17.4	16.6

* Tr. = treated, and Ck. = check, or control plants not treated.

seen that the catalase values are higher for the treated lots in each of the four tests and for each determination in each test. For invertase the differences are not so great, but it seems certain that the treatment increased the invertase activity of the tissue. The amylase values, however, fail to show an increase of the treated over check lots, except possibly at the longer periods, that is, on the fifth and sixth days after treatment. Turning now to the data for the twig-bark as shown in Table II, we find again in general an increase in catalase, but the increases were much smaller with the twig-bark than with the bud tissue, and, in the November 16 treatment, practically no differences at all were observed even in the sixth-day sample. The invertase values also show evidence of increase as a result of treatment; however, two exceptions are noted: on the second day for the treatment of November 16, and on the fourth day for December 9. The amylase values are not higher for the treated lots than for the checks, and in fact,

are distinctly lower in several comparable pairs. Further work would be necessary to determine conclusively the effect of the chemical treatment upon the amylase activity of the twig-bark.

TABLE II

EFFECT OF ETHYLENE CHLORHYDRIN TREATMENT UPON ENZYME ACTIVITIES OF TWIG-BARK TISSUE

Date treated 1931	Days after treatment	Catalase cc. O_2 per 0.1 g.		Amylase cc. $KMnO_4$ per 0.03 g.		Invertase cc. $KMnO_4$ per 0.2 g.	
		Tr.*	Ck.	Tr.*	Ck.	Tr.*	Ck.
Nov. 10	3	7.2	6.7	33.8	35.8	14.6	10.2
	5	5.8	3.9	23.7	34.4	18.5	13.4
Nov. 16	2	1.3	2.0	21.8	31.6	5.6	10.3
	4	3.3	3.4	22.0	36.0	**	**
	6	4.1	3.2	22.2	28.1	11.8	9.5
Dec. 2	2	4.7	3.2	31.4	31.2	19.2	10.5
	4	3.7	2.7	27.7	27.8	11.8	8.1
	6	4.4	3.8	32.6	31.8	9.2	7.3
Dec. 9	4	5.1	4.4	30.9	33.4	8.0	10.8
	6	6.5	5.6	34.2	33.0	12.9	9.4

* Tr. = treated, and Ck. = check, or control plants not treated.
** Determination lost.

TABLE III

EFFECT OF ETHYLENE CHLORHYDRIN TREATMENT UPON ENZYME ACTIVITIES OF STEM-BARK TISSUE

Date treated 1931	Days after treatment	Catalase cc. O_2 per 0.1 g.		Amylase cc. $KMnO_4$ per 0.03 g.		Invertase cc. $KMnO_4$ per 0.2 g.	
		Tr.*	Ck.	Tr.*	Ck.	Tr.*	Ck.
Nov. 10	3	3.5	3.5	31.2	27.5	9.8	7.2
	5	6.6	4.0	33.4	35.3	9.0	7.8
Nov. 16	2	0.7	1.4	31.6	37.9	4.7	4.2
	4	2.1	1.4	33.7	31.2	4.6	7.5
	6	3.5	1.9	23.9	35.3	7.0	5.0
Dec. 2	2	2.0	2.2	33.2	34.5	3.8	8.8
	4	3.1	2.6	35.0	34.4	3.2	5.3
	6	3.9	3.2	36.5	31.0	13.8	8.5
Dec. 9	4	4.4	4.4	32.4	33.4	7.4	8.9
	6	7.4	5.5	35.5	36.4	12.7	12.6

* Tr. = treated, and Ck. = check, or control plants not treated.

The enzyme results for the stem-bark tissue are given in Table III. The only differences shown are in the catalase values on the fifth and sixth days after treatment, and even in these cases, the increase due to treatment is small. The invertase and amylase values for treated and check

lots do not furnish a safe conclusion regarding the effect of the treatment upon the activity of these enzymes.

The results described above refer to the effects of the treatment upon the enzyme activity when the plants were treated by the chemical vapors, and when tissue was taken subsequently from the treated plants for the enzyme tests. The question arises whether these effects were produced by the chemical acting directly upon the enzymes or acting indirectly upon them, that is, acting first upon the living tissue which then influenced the enzyme activity. Information on this point was obtained by adding varying amounts of ethylene chlorhydrin directly to a preparation of twig-bark tissue. The tests were carried out in the same manner as has been described for the tissue which was obtained in the experiments in which entire plants

TABLE IV

DIRECT EFFECT OF ETHYLENE CHLORHYDRIN UPON ENZYMES OF LILAC STEM-BARK

Catalase		Amylase		Invertase	
Chlorhydrin added, g.	Oxygen released, cc.	Chlorhydrin added, g.	cc. $KMnO_4$	Chlorhydrin added, g.	cc. $KMnO_4$
None	5.6	None	25.7	None	13.0
"	5.7	"	25.9	"	11.9
"	5.4	0.04	24.4	0.01	13.0
0.04	5.3	0.11	26.2	0.03	12.2
0.08	5.1	0.33	25.5	0.08	11.9
0.16	5.3	0.97	27.2	0.24	13.0
0.33	4.7	2.92	26.1	0.73	13.1
0.65	4.1	8.77	27.8	2.19	13.9
1.37	3.1			6.58	13.2
2.74	2.1				

were treated. The results for catalase, amylase, and invertase are shown in Table IV. It is seen that catalase activity was not increased by the addition of ethylene chlorhydrin, and, in fact, that when amounts greater than about one gram per 100 cc. of reaction-mixture were added, the catalase activity was strongly retarded. The results with amylase and invertase show practically no influence of ethylene chlorhydrin, even when rather large amounts were added.

Recapitulating the results of the enzyme measurements, it was found that the ethylene chlorhydrin treatment induced an increase in catalase, and a somewhat less distinct increase in invertase, but that the effect upon amylase was so small as to leave the question undecided as to whether increases or decreases resulted. The effects could not be ascribed to any direct effect of the chemical upon the enzymes themselves, but rather to the indirect effect of the chemical upon the life processes of the intact plant.

TABLE V

EFFECT OF ETHYLENE CHLORHYDRIN TREATMENT UPON THE RESPIRATION OF LILAC TWIGS. TWIGS DETACHED FROM PLANT

Date treated 1931	Day after treatment	Tr. or ck.*	Wt. of twigs, g.	Area, sq. cm.	Milligrams of CO_2 per hour		
					Per twig	Per 100 g.	Per 100 sq. cm.
Nov. 16	First	Tr. Ck.	13.16 13.24		0.50 0.41	15.1 12.3	
	Second	Tr. Ck.	13.16 13.24		0.39 0.31	12.0 9.4	
	Sixth	Tr. Ck.	6.76 7.65		0.24 0.18	17.5 11.5	
	Seventh	Tr. Ck.	6.76 7.65		0.20 0.16	16.3 11.5	
Dec. 2	Second	Tr. Ck.	5.18 5.17		0.52 0.22	29.6 12.9	
	3+4**	Tr. Ck.	3.26 3.33		0.22 0.19	14.4 11.5	
	4+5**	Tr. Ck.	5.84 4.54		0.22 0.16	11.4 10.7	
Dec. 9	First	Tr. Ck.	4.48 4.33	34.9 34.6	0.46 0.31	30.5 21.3	3.91 2.66
	Second	Tr. Ck.	3.68 3.66	30.1 29.9	0.46 0.35	37.3 28.2	4.55 3.49
	3+4**	Tr. Ck.	5.61 5.19	36.3 36.6	0.31 0.16	16.7 11.1	2.57 1.57
	Fifth	Tr. Ck.	3.92 4.06	29.5 33.0	0.32 0.14	24.7 10.7	3.29 1.31
	Sixth	Tr. Ck.	3.83 4.00	32.2 38.5	0.35 0.13	27.4 9.9	3.26 1.03
Dec. 15	First	Tr. Ck.	7.32 6.42	42.84 43.31	0.78 0.32	27.8 15.0	4.75 2.22
	Second	Tr. Ck.	5.13 5.05	36.76 36.74	0.63 0.26	37.0 15.4	5.11 2.11
	Third	Tr. Ck.	5.24 5.15	34.91 35.70	0.55 0.19	31.4 11.2	4.71 1.61
	4+5**	Tr. Ck.	5.30 5.03	35.02 38.62	0.39 0.23	21.8 14.0	3.30 1.82
	Sixth	Tr. Ck.	4.49 5.14	27.63 30.67	0.58 0.27	25.8 10.6	4.20 1.77

* Tr. = treated, and Ck. = check, or control plants not treated.

** 3+4 means respiration during third and fourth days; 4+5 means respiration during fourth and fifth days.

EFFECT OF TREATMENT ON RESPIRATION

The results of the respiration measurements are shown in Tables V and VI. The data for the twigs that had been detached from the plants are given in Table V. From columns 6, 7, and 8 it is seen that three different bases were used for expressing the respiration values, i.e., per twig, per 100 grams of tissue, and per 100 sq. cm. of area. The twigs were all about 10 cm. long and all had the same number of buds but they varied in thickness from about 0.22 cm. to about 0.52 cm. The surface area was determined by measuring the diameter of the twig at three places and

TABLE VI

EFFECT OF ETHYLENE CHLORHYDRIN UPON THE RESPIRATION OF LILAC TWIGS. TWIGS NOT DETACHED FROM PLANT

Date treated 1931	No. twigs	Total weight, g.		Total area, sq. cm.		Days after treatment	Average mg. CO_2 per hour			
							Per 100 g.		Per 100 sq. cm.	
		Tr.*	Ck.	Tr.*	Ck.		Tr.*	Ck.	Tr.*	Ck.
Dec. 2	2	4.05	3.20	22.8	25.2	2nd	27.4	18.5	4.81	2.64
						3+4**	12.8	10.6	2.21	1.51
						5th	22.5	14.6	3.93	2.11
Dec. 9	2	5.33	4.99	28.8	27.6	1st	20.5	15.1	3.74	2.73
						2nd	18.9	13.9	3.49	2.50
						3+4**	8.7	7.9	1.67	1.42
						5th	9.6	8.8	1.78	1.58
						6th	12.5	7.1	2.30	1.26
Dec. 15	3	7.64	6.64	37.9	30.1	1st	32.2	12.0	6.39	1.87
						2nd	35.0	9.5	6.98	1.50
						3rd	34.7	11.0	6.86	1.77
						4+5**	36.3	14.4	7.26	2.03
						6th	35.6	18.5	7.15	2.76

* Tr. = treated, and Ck. = check, or control plants not treated.

** 3+4 means respiration during third and fourth days; 4+5 means respiration during fourth and fifth days.

multiplying the average circumference by the length of the twig. When calculated on the basis of the weight of the twigs, there was a tendency for smaller twigs to give a higher calculated rate than larger ones. This probably was due to the fact that with smaller twigs a relatively larger proportion of the weight consists of the actively respiring bark and bud tissue. But in spite of these difficulties regarding a suitable basis for expressing respiration rates, Table V shows that the increases in respiration due to chemical treatment were about the same for all three methods of calculation. The November and early December treatments induced an increase of about 20 to 50 per cent in the rate of production of carbon dioxide by the twigs, and the December 15th test showed increases of about 100 to 150 per cent.

The measurements of the respiration of twigs that remained intact upon the plant are shown in Table VI. The problem of obtaining treated and check twigs of the same size for comparison was more difficult with attached than with detached twigs. The weight of the twig could be determined only at the end of the experiment, and, unfortunately, as shown in Table VI, it was found that in each test the check twigs averaged smaller than the treated ones. Consequently in Table VI, the per twig basis is omitted from consideration, and the carbon dioxide values are given only per 100 grams, and per 100 sq. cm. of area. The plants were in the greenhouse during the time in which measurements of the respiration of attached twigs were made, and, therefore, the respiration tests could not be made at constant temperature. This may account for some of the fluctuation in the respiration rate from day to day. But a comparison of the treated and check lots under comparable conditions, i.e., on the same day, shows increases of treated over check values. These increases range from about 10 to about 200 per cent, or even more.

EFFECT OF TREATMENT ON CHEMICAL COMPOSITION

The results of the chemical analyses of the tissues at intervals after treatment are shown for the buds, twig-bark, and stem-bark in Tables VII, VIII, and IX. The moisture (see column 4) was found to increase in the buds promptly after treatment, and, to a less extent, in the twig-bark, but hardly at all in the stem-bark. The soluble nitrogen values are shown in column 5 in Tables VII, VIII, and IX. The treated lots showed increases, especially in the bud and twig-bark tissue, and to a much less extent in the stem-bark. The results for sugars are shown in columns 7, 8, and 9 in Tables VII, VIII, and IX. The sucrose values by the invertase method are much lower than those by acid hydrolysis, showing the presence of some other substance besides sucrose that was hydrolyzed by acid at room temperature. But the sucrose values by either method show definite decreases as a result of the treatment. This is true of all three types of tissue, the differences between treated and check decreasing in the order of buds, twig-bark, and stem-bark. The direct reducing sugar values were less consistent in this respect but on the whole were somewhat lower for the treated than for the check. Probably these low sugar values in the treated lots were caused by the increased respiration induced by the treatment. The starch and polysaccharide values are shown in columns 10 and 11 in Tables VII, VIII, and IX. The starch percentage was low showing that most of the alcohol-insoluble acid-hydrolyzable substances were represented by non-starch polysaccharides. The values for these two types of storage products are difficult to interpret. We might expect a decrease in starch since it furnished a source of the sugar that was used up in respiration, but the data do not give conclusive evidence of loss of either starch

TABLE VII

EFFECT OF ETHYLENE CHLORHYDRIN TREATMENT UPON THE CHEMICAL COMPOSITION OF BUD TISSUE

Date treated 1931	Tr. or ck.*	Days after treatment	Per cent H_2O	Per cent of the dry weight						
				Nitrogen		Red. sugar	Sucrose		Polysacch.	Starch
				Sol.	Insol.		By acid inversion	By invertase		
Nov. 10	Tr.	3	56.8	0.24	1.81	0.64	3.79	**	13.2	1.93
	Ck.	3	53.2	0.17	1.76	1.22	7.18	2.24	14.7	2.96
	Tr.	5	58.1	0.24	1.76	2.05	4.07	0.83	12.7	1.72
	Ck.	5	54.5	0.18	1.69	1.56	6.89	2.29	12.9	3.03
Nov. 16	Tr.	2	53.3	0.18	1.49	1.03	**	3.95	16.1	2.92
	Ck.	2	52.6	0.18	1.65	1.32	**	3.79	16.4	3.64
	Tr.	4	55.2	0.21	1.49	1.55	5.73	1.37	13.9	2.18
	Ck.	4	52.8	0.15	1.69	1.58	5.47	**	15.1	2.44
	Tr.	6	58.1	0.22	1.60	0.97	5.19	1.87	11.6	2.29
	Ck.	6	55.3	0.12	1.65	1.47	6.30	3.80	12.5	2.75
Dec. 2	Tr.	2	52.5	0.22	1.58	1.34	8.36	3.60	16.5	1.65
	Ck.	2	46.6	0.17	1.60	1.81	8.07	4.82	16.1	1.83
	Tr.	4	52.6	0.22	1.69	0.86	7.00	3.17	13.2	2.19
	Ck.	4	49.9	0.16	1.61	1.48	7.64	4.18	13.5	1.86
	Tr.	6	58.5	0.32	1.63	2.62	5.17	2.56	12.2	2.16
	Ck.	6	52.5	0.21	1.57	3.04	6.92	3.03	12.4	2.18
Dec. 9	Tr.	4	54.7	0.21	1.79	2.37	5.79	2.81	12.4	1.91
	Ck.	4	52.4	0.19	1.75	2.55	7.14	3.28	13.2	1.68
	Tr.	6	57.6	0.20	1.80	5.07	5.23	1.91	13.3	2.12
	Ck.	6	52.3	0.13	1.72	4.06	6.64	3.36	13.1	1.86

* Tr. = treated, and Ck. = check, or control plants not treated.
** Insufficient material for determination.

TABLE VIII

EFFECT OF ETHYLENE CHLORHYDRIN TREATMENT UPON THE CHEMICAL COMPOSITION OF TWIG-BARK TISSUE

Date treated 1931	Tr. or ck.*	Days after treatment	Per cent H_2O	Per cent of the dry weight							
				Nitrogen		Red. sugar	Sucrose		Polysacch.	Starch
				Sol.	Insol.		By acid inversion	By invertase		
Nov. 10	Tr.	3	45.3	0.15	1.21	2.76	3.10	1.08	19.5	3.58
	Ck.	3	45.6	0.09	1.30	2.74	4.90	2.08	19.6	4.68
	Tr.	5	50.0	0.18	1.39	2.82	2.01	0.26	16.7	4.56
	Ck.	5	46.8	0.08	1.39	2.99	4.34	2.13	18.2	4.77
Nov. 16	Tr.	2	43.7	0.10	1.08	2.96	3.13	3.28	20.7	2.41
	Ck.	2	45.6	0.09	1.22	3.20	4.23	2.81	19.8	3.11
	Tr.	4	45.0	0.13	1.03	2.55	2.97	1.90	19.3	3.51
	Ck.	4	45.8	0.10	1.19	2.92	5.10	2.70	17.8	4.16
	Tr.	6	45.2	0.14	1.06	2.73	2.10	1.16	17.4	3.84
	Ck.	6	43.1	0.11	1.24	3.04	3.61	2.05	15.2	3.61
Dec. 2	Tr.	2	43.3	0.14	1.30	2.97	4.15	2.84	17.8	3.83
	Ck.	2	44.9	0.10	1.39	3.86	4.46	2.09	18.1	4.88
	Tr.	4	43.3	0.15	1.16	3.42	3.22	1.42	17.3	2.75
	Ck.	4	41.3	0.10	1.28	3.36	5.70	3.10	17.2	3.24
	Tr.	6	45.7	0.19	1.11	2.55	3.67	1.25	18.8	3.59
	Ck.	6	44.8	0.13	1.30	2.79	5.43	2.44	17.0	2.95
Dec. 9	Tr.	4	45.7	0.15	1.46	3.38	5.55	3.10	16.8	2.60
	Ck.	4	44.7	0.10	1.69	3.60	6.60	3.50	16.0	1.97
	Tr.	6	45.6	0.16	1.44	3.71	4.05	2.02	18.3	3.42
	Ck.	6	44.9	0.11	1.51	4.04	5.35	2.83	16.6	2.12

* Tr. = treated, and Ck. = check, or control plants not treated.

TABLE IX

EFFECT OF ETHYLENE CHLORHYDRIN TREATMENT UPON THE CHEMICAL COMPOSITION OF STEM-BARK TISSUE

Date treated 1931	Tr. or ck.*	Days after treatment	Per cent H_2O	Per cent of the dry weight						
				Nitrogen		Red. sugar	Sucrose		Polysacch.	Starch
				Sol.	Insol.		By acid inversion	By invertase		
Nov. 10	Tr.	3	47.8	0.13	0.95	3.18	4.18	2.88	18.9	4.98
	Ck.	3	46.5	0.08	1.26	3.15	5.83	3.93	19.9	5.13
	Tr.	5	48.2	0.17	1.26	2.15	3.01	2.28	18.9	4.97
	Ck.	5	47.0	0.09	1.41	2.87	4.41	2.32	18.[illegible]	5.10
Nov. 16	Tr.	2	47.8	0.10	0.96	3.57	5.62	4.23	17.9	3.62
	Ck.	2	48.1	0.08	1.17	3.64	5.59	4.22	18.9	4.23
	Tr.	4	44.3	0.09	0.93	3.27	4.54	3.00	18.5	3.89
	Ck.	4	44.5	0.09	0.99	3.34	4.28	2.99	18.7	3.68
	Tr.	6	47.1	0.11	1.06	2.97	4.32	2.26	19.8	3.76
	Ck.	6	47.6	0.09	1.07	3.02	5.07	3.23	19.3	3.76
Dec. 2	Tr.	2	46.9	0.10	1.18	2.94	6.13	2.81	18.4	2.37
	Ck.	2	47.2	0.10	1.18	3.41	6.11	3.94	16.7	2.24
	Tr.	4	46.3	0.12	1.16	3.40	6.12	4.48	17.6	2.22
	Ck.	4	46.5	0.09	1.19	3.64	6.37	4.50	15.4	2.02
	Tr.	6	48.3	0.13	1.16	3.17	5.68	3.40	17.9	2.79
	Ck.	6	48.9	0.12	1.18	3.52	6.10	4.36	17.3	2.46
Dec. 9	Tr.	4	42.6	0.10	1.47	4.03	5.49	3.42	16.9	1.81
	Ck.	4	42.2	0.08	1.58	4.12	4.98	3.80	16.4	1.58
	Tr.	6	43.9	0.08	1.43	3.36	4.38	2.31	16.3	1.73
	Ck.	6	45.6	0.05	1.48	3.46	4.70	3.20	17.2	1.64

* Tr. = treated, and Ck. = check, or control plants not treated.

or other insoluble hydrolyzable polysaccharides. These substances may have been lower in the bud tissue of the treated lots, but no differences in the twig-bark or stem-bark are shown. The bark contained a small amount of green tissue and it is possible that photosynthesis may have occurred. The most consistent behavior of the starch was the decrease in the amount present in the tissue during the course of the experiment, being much higher at the beginning, November 10, than at the end, December 9. However, this change was true of both treated and check tissues, the extent of the change being somewhat less for treated buds than for the checks, but being nearly identical for the stem-bark of treated and check tissue.

DISCUSSION

The changes which occurred most quickly after the treatment were the increases in respiration of twigs and in moisture of buds. Even on the first day after the end of the treatment the twig respiration was observed to be higher in the treated lots than in the checks, and, although data on the moisture of buds were not available until the second day, an increase was found to have occurred at that time. On the third and fourth days the increases in catalase and soluble nitrogen and the decrease in sucrose became evident in the buds and twig-bark. Any changes which occurred in the stem-bark were smaller and occurred later than in buds and twig-bark.

The failure to find a pronounced change in amylase activity was unexpected in view of previous results with potatoes (4), and of the earlier experiments of Howard (7, p. 24) with the treatments of dormant woody plants with ethyl ether. However, Howard studied the splitting of starch into products not giving a blue color with iodine, while in the present experiments the saccharogenic properties of the amylase were measured. The present status of the amylase response in the treatment of lilacs with ethylene chlorhydrin is uncertain. Tests utilizing different methods of preparing the tissue and of carrying out the tests may be necessary to show the facts. At present it does not appear that the amylase factor is very important in the effect of the treatments in inducing growth.

The effect of ethylene chlorhydrin upon the respiration of lilac twigs is similar to that observed by Howard (7) in the etherization experiments with apple twigs. We confirm his observation that the increase in respiration can become observable and even extensive on the first day after the end of the treatment.

The *decrease* in the sugar content of the treated twigs is similar to the results obtained by Müller-Thurgau and Schneider-Orelli (8) in a study of the effect of the warm-bath treatment of lilacs. Niethammer (9), however, breaking the dormancy of lilacs by means of frost and by drying the twigs, found that the treatments caused an *increase* in total sugar. These

differences in the response of the lilac to different treatments, all of which break dormancy, may find their explanation in differences in the effect upon the rate of respiration.

The results with lilac twigs showing a loss of sucrose as a result of the treatment with ethylene chlorhydrin are in sharp contrast to those previously described (3) for potatoes in which an increase in sucrose was obtained. Perhaps this difference in behavior to the same chemical is related to the starch content of the two species. In potatoes the starch percentage is high enough to permit the maintenance of a high sugar supply in spite of the high respiration, while in the lilac the starch content is too low to permit the sugar formation to keep pace with its loss in respiration.

Another difference in the behavior of sugar in dormancy studies is noted when comparison is made with the results of Gardner (6) who studied the changes induced by low temperature in shortening the rest period of Bartlett pear shoots. In his experiment the condition which was favorable for breaking dormancy resulted in an increase of sugars, both hexoses and sucrose. Here again, the differences in sugar content of the tissues in Gardner's experiments and in the present experiment are probably related to differences in respiration rate, sugar accumulation in the pear twigs being induced by low temperature and low respiration, and sugar loss in the treated lilac twigs by high respiration.

SUMMARY

1. At four different stages of the rest period, pot-grown plants of lilac (*Syringa vulgaris* L., var. Charles X) were treated with vapors of ethylene chlorhydrin ($ClCH_2CH_2OH$), and were placed in the greenhouse. On the second or third day after treatment, and thereafter at intervals of two days, branches were removed for determinations of enzyme activity and chemical composition. The checks consisted of lilac plants from the same lot subjected to the same procedure except that they were not exposed to chemical vapors. Sampling was discontinued when the treated plants showed visible signs of swelling of buds.

2. Three types of tissue were obtained: bud, tissue external to the woody cylinder, called twig-bark if from twigs of the current season's growth, and stem-bark if from stems of greater age.

3. Catalase increases in the treated tissue were large in the bud tissue, less in twig-bark, and were much less and occurred later in the stem-bark. Invertase increased somewhat in the buds of treated plants, only slightly in the twig-bark and not at all in the stem-bark. No extensive increase in amylase activity was shown in any type of tissue.

4. The most noteworthy chemical changes observed were: increases in moisture of buds, increases in soluble nitrogenous substances, and decreases in sugars, especially sucrose.

5. Measurements of the respiration of twigs, either while attached to the plants or after removal from them, showed that increases in the treated twigs were observable as early as the first day after the end of the treatment. The amount of this increase varied from about 20 per cent in the early stages of the rest period to more than 100 per cent in the later stages.

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